Development of the capacity of the gut-associated immune system of perinatal lambs to respond to parasitic antigens

A thesis submitted for the degree of

Doctor of Philosophy

of the Australian National University

by

Reji Francis Paalangara

Developmental Physiology Group
Division of Molecular Medicine
John Curtin School of Medical Research
The Australian National University
Canberra, ACT
Australia

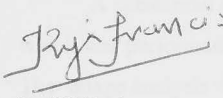
April 2000

For,

SUJA EMIL & NAOMI

STATEMENT OF ORIGINALITY

The work described in this thesis is original and was carried out by me under the supervision of Dr. Peter J McCullagh in the Division of Molecular Medicine at the John Curtin School of Medical Research, Australian National University. With the exception of worm count and faecal count assays which were done at the McMaster laboratory, CSIRO, Sydney, all of the contents of this thesis are the original work of the author and have neither been presented nor are currently being presented for any other degree.



Reji F Paalangara

ACKNOWLEDGEMENTS

I am indebted to my supervisor Dr. Peter J McCullagh for the expert guidance and the encouragement he has given me all along for the completion of this project. It is with immense pleasure that I express my sincere gratitude to him.

I am indebted to Dr. Susan J McClure for her valuable advice, guidance and unstinted help rendered during the course of the work.

I wish to acknowledge with gratitude the consistent help that Bernie Barancewicz provided to me in the operating theatre. Besides, I would also like to thank Karen King, Rhonda Davey and Gordon for all their help and co-operation rendered throughout the study.

A substantial component of the research work involved image analysis which was performed at the FishBowl, Research School of Biological Sciences. The image analysis was made possible because of the timely help provide by David Vowels, Sally Stowes, Eldon Ball and other staff members there. My sincere thanks to them.

Sabine Gruninger and Geoffrey Osborne were of great help with the flow cytometry studies and I would like to express my gratitude to them. The help rendered by staff from histology and photography is gratefully acknowledged.

I am extremely grateful to the Australian National University for providing me the ANU PhD Scholarship which made it possible for me to undertake this project.

My friend Om Prakash has been a tremendous source of inspiration throughout the difficult times. I also appreciate the encouragement and help given by all our friends from Sydney which helped me keep my moral up.

This work would not have been possible but for my wife and children's patience and sacrifice through all the uncertain times. The blessings of my family in India have always kept my hopes high.

ABSTRACT

The mucosal immune system is, in terms of cell numbers, larger than the remainder of the immune system. The ileal Peyer's patches of the neonatal sheep function as the primary lymphoid organ for the production of B lymphocytes. Apart from this role, the ileal Peyer's patches, together with the Peyer's patches from other regions of the gut can function as secondary lymphoid tissue responsible for mounting local immune responses.

The nature of the immune responses mounted by the ruminant gut in response to parasites has not been clearly defined. The aim of this project was to elucidate information about the various forms of response of the young lamb to parasite entry into the gut. To achieve this, one group of foetal lambs received an orally administered extract prepared from larval *Trichostrongylus colubriformis* some 50 days before birth. A control group of lambs received normal saline at the same age. Each group of lambs was subsequently challenged with live *Trichostrongylus colubriformis* larvae 4-6 weeks after birth. All the lambs were sacrificed 9 weeks after receiving the parasite and a detailed comparison of the two groups was undertaken using a large set of parameters (22 markers). The goal was to identify any modifications of the response of the gut to the parasite, which were attributable to exposure *in utero* and so to infer the effects of that exposure on subsequent responsiveness.

Some substantial differences were evident between the responsiveness to *Trichostrongylus colubriformis* between the two groups of lambs. Whilst the extreme paucity of pre-existing information about the likely consequences for later responsiveness of premature antigenic exposure of the gut preclude formulation of any hypotheses for testing, the results of this experiment were supportive of the proposition that subsequent postnatal responsiveness had been reduced by antigenic exposure. Notwithstanding the differences observed in many parameters of responsiveness, there was no significant differences in the efficiency with which the two groups eliminated parasites or constrained their development.

The distribution and frequency of occurrence of $CD4^+$ and $CD8^+$ T cells were significantly different between the two groups of lambs. It appeared likely that these differences reflected different migratory patterns, presumably of

specific antigenically sensitised subpopulations in response to challenge. Eosinophil levels, both in the gut tissue and in circulating blood, which have been reported to correlate directly with parasite resistance, were higher in control lambs. Another suggested correlate of resistance to parasite, goblet cell frequency, was also significantly greater in the control group. The *in utero* antigenic exposure also brought about significant changes in the $CD1b^+$ and $CD1c^+$ dendritic cell population of the infected gut among the two groups of lambs. Interleukin expression within the gut was also significantly altered by the prenatal oral antigenic exposure. The *in utero* antigen-exposed lambs had significantly elevated levels of TNF-α and TGF-β within the gut in comparison with control lambs. In contrast, control lambs had significantly higher levels of IL-1 β and high levels of IL-2 in comparison with *in utero* antigen-exposed lambs. The production of neuron specific Enolase, which has been proposed to be an indicator of lymphocyte differentiation, was significantly greater in control lambs.

CONTENTS

Abbreviations used

Ag:	Antigen
APC:	Antigen presenting cells
BSA:	Bovine serum albumin
ConA:	Concanavalin A
DC:	Dendritic cell
DCs	Dendritic cells
EDTA:	Ethylene Diamine Tetra Acetic acid
IEL:	Intraepithelial lymphocytes
Ig:	Immunoglobulin
IL:	Interleukin
iNos:	Inducible nitric oxide synthase
IPP:	Ileal Peyer's patch
JPP:	Jejunal Peyer's patch
LPS:	Lipopolysaccharide
MAb:	Monoclonal antibody
MLN:	Mesenteric lymph node
MHC:	Major histocompatibility complex
mRNA:	Messenger ribonucleic acid
NS:	Not significant statistically
NSE:	Neuron specific Enolase
PBS:	Phosphate buffered saline
Th	T helper
TNF:	Tumor necrosis factor
TGF:	Transforming growth factor
S.E.:	Standard error
Sig:	Statistically significant

INTRODUCTION

A substantial number of the antigens encountered by the immune system gain access to the body via a mucosal surface such as the intestine or respiratory tract. To tackle this massive influx of antigens the various mucosae have developed a large and complex immune apparatus, which is anatomically and functionally distinct from that found elsewhere in the body. The mucosal immune system comprises that lymphoid tissue associated with the internal surfaces of the body, namely the gastrointestinal tract, the upper and lower respiratory tract and the urogenital tract. The lymphoid tissues associated with the mucosal surfaces comprise the largest compartment of the immune system, with a greater number of lymphocytes present in the mucosal immune system than in all other parts of the immune system combined (Langkamp-Henken *et al.*, 1992; Mowat & Viney, 1997).

The gut associated lymphoid tissue comprises the Peyer's patches, mesenteric lymph nodes and a large number of lymphoid cells scattered throughout the lamina propria and epithelium of the intestine. Peyer's patches are organised areas of lymphoid tissue found in the gastrointestinal tract. The majority of Peyer's patches have the anatomical features of a classical secondary lymphoid organ, with clearly defined T and B cell-dependent areas. They are located submucosally, separated from the intestinal lumen by a single layer of cuboidal epithelium cells, the follicle associated epithelium. The epithelial layer overlying the Peyer's patches is composed of a heterogeneous population of cells. In addition to conventional enterocytes, the epithelium contains many lymphoid cells as well as a unique population of specialised epithelial cells called M cells. The primary function of these M cells appear to be the uptake and transport of antigens into the Peyer's patches (McGowan *et al.*, 1997; Mowat & Viney, 1997).

The mesenteric lymph nodes form another type of aggregated lymphoid tissue associated with the gut immune system. They are identical in overall structure to other peripheral lymph nodes. These mesenteric lymph nodes receive the lymphatic vessels which drain the Peyer's patches and lamina propria of the gut.

In addition to the organised lymphoid tissues namely the Peyer's patches and the mesenteric lymph nodes, the villus/ crypt units of the intestine contain large numbers of scattered lymphocytes. These lymphocytes are distributed throughout both the epithelium and the deeper layers of the lamina propria of the gut. In contrast to the Peyer's patches and the mesenteric lymph nodes, which act as inductive sites, epithelial lymphocytes are thought to represent the effector arm of the local immune response in the gut (Mowat & Viney, 1997). The lamina propria contains most components of the immune system, with larger numbers of B cells, plasma cells, macrophages, dendritic cells and T cells. Around 10-15 % of the cells in the gut epithelium are lymphocytes of which in excess of 90 % are T cells (Mowat & Viney, 1997). There is thought to be considerable cross-talk between the epithelial cells and the lymphocytes within the mucosa of the gastrointestinal tract.

A unique feature of the mucosal immune system is that lymphocytes activated at mucosal sites such as the Peyer's patches and the mesenteric lymph nodes will preferentially recirculate back to mucosal surfaces rather than to the peripheral immune system. Lymphocyte trafficking within the mucosal immune system is regulated via the expression of specific adhesion molecules on lymphocytes and their corresponding ligand molecules on the local vascular endothelium (McGowan *et al*., 1997).

Gut antigens can enter the mucosa by a number of mechanisms. The M cells are covered by folds and crevices to which microorganisms and other luminal antigens can bind preferentially. However, M cells cannot process antigens and it believed that the antigen entering them is delivered intact to professional antigen presenting cells (Mayer, 1997; Mowat & Viney, 1997). Gut luminal antigens can also enter the mucosa via conventional enterocytes, either by paracellular routes or via the enterocytes themselves (Mowat & Viney, 1997). Any antigen taken up through the M cells or the enterocytes is delivered to professional antigen presenting cells which are abundant both in association with the follicle associated epithelium and in the underlying areas of the Peyer's patches. These antigen presenting cells are thought to sensitise T cells in the conventional manner (Mowat & Viney, 1997). In this context it is necessary to point out that dendritic cells associated with the mucosal immune system may

differ from their peripheral counterparts in phenotype and functions. The presentation of antigens by these antigen presenting cells could have immunological consequences which differ from those following presentation of antigen by cells in non-mucosal locations (Kelsall & Strober, 1996; Viney *et al.*, 1998). The eventual outcome of the immune response is likely to be dependent on the level of inflammation and co-stimulatory activity induced by the antigen. It is hypothesised that inert gut antigens such as soluble proteins may induce systemic T cell tolerance because they are delivered to the immune system by antigen-laden dendritic cells which exit from the gut without acquisition of significant co-stimulatory activity (Mowat & Viney, 1997). Another possible route through which antigen uptake occurs from the gut is via the epithelial cells. This mode of antigen presentation is thought to preferentially stimulate suppressor $CD8^+$ T cells within the gut.

The bulk of the body's IgA antibodies, as well as the total pool of immunoglobulins are produced by the plasma cells in the mucosal lamina propria (Lamm, 1998). Mucosal IgA is very effective in preventing the adhesion and absorption of antigen into the gut mucosa. Because of the enormous amounts of IgA produced in the intestine, it is often assumed that lamina propria $CD4^+$ T cells are activated T helper type 2 (Th2) cells which regulate the differentiation of IgA-producing B cells. This hypothesis is contentious and many researchers consider it to be an oversimplification of events occurring in the gastrointestinal tract. The current belief is that there may be no inherent bias towards one or other T helper subset in the lamina propria, although Th2 type cytokines could normally predominate in the absence of inflammation (Mowat & Viney, 1997).

Evidence is gradually emerging that the intestine may be an alternative primary lymphoid organ for both B and T lymphocytes. The intestinal epithelium is a site in which some T cells differentiate extrathymically (Mowat & Viney, 1997). There is clear evidence that the Peyer's patches of the distal small intestine are the site of primary B-cell development in some species such as the sheep (Griebel & Hein, 1996).

Additional features of the gut immune system in the sheep: The Peyer's patch plays an important role as a primary B-lymphoid organ in some species of animals. Much of the physiological evidence for this has come from

experimental studies in sheep. In sheep and other ruminants the Peyer's patch tissue is unequally distributed along the small intestine, with more occurring in the ileum than in the jejunum. The ileum contains 80-90 % of the total mass of the Peyer's patch tissue (Reynolds & Morris, 1983). The ileal Peyer's patch occurs as a single continuous aggregation of lymphoid follicles, commencing at or near the ileo-caecal junction and extending cranially for a variable distance. The ileal Peyer's patch is most prominent in young animals in which it may extend for up to two metres in length. In contrast, the jejunal Peyer's patch occurs as multiple, discrete accumulations of lymphoid follicles.

The development of the Peyer's patches of sheep starts in foetal life. In this aspect the sheep differs from other laboratory animals such as the rat, mouse or rabbit where there is very little prenatal development of the Peyer's patches (Griebel & Hein, 1996). In these other species Peyer's patch development occurs during the first two weeks of postnatal life. The jejunal and ileal Peyer's patches of sheep have a very different life history after birth. The ileal Peyer's patches reach their maximum size early in life and then start to involute in a similar manner to the thymus. In the young lamb the total mass of the ileal Peyer's patches is greatest at 2-3 months after birth. Within 18 months, complete involution of the ileal Peyer's patches has occurred (Reynolds & Morris, 1983). In contrast, the jejunal Peyer's patches persist throughout the life of the sheep, although the density of the follicles in each patch may decline with advancing age.

The ileal and jejunal Peyer's patches comprise individual lymphoid follicles each of which is surrounded by a capsule of connective tissue. The number and organisation of follicles differ between the ileal and jejunal Peyer's patches. In the young lamb the entire stretch of the ileal Peyer's patches contains about 100,000 individual follicles that are larger than those in the jejunum (Reynolds & Morris, 1983). The jejunal Peyer's patches, on the other hand, contain a mere one tenth of the number of follicles found in the ileal Peyer's patches. In the ileum, the follicles are densely packed together with only scant interfollicular T-cell areas. In the jejunum the follicles are more widely dispersed and are separated by prominent interfollicular regions rich in mature lymphocytes including T cells (Hein *et al.*, 1989).

The composition of the ileal and jejunal Peyer's patches of sheep diverges soon after birth. The ileal Peyer's patch follicle is composed almost exclusively of B cells (< 1 % T cells). The majority of these B cells (> 95 %) express surface IgM. There are a few plasma cells (IgM^+) and a few isotype-switched and $CD5^+$ B cells (Griebel & Ferrari, 1995). At birth, the jejunal Peyer's patches also contain predominantly surface IgM^+ B cells but the lymphoid composition of these follicles changes rapidly after birth and becomes more heterogeneous. Within a few weeks, the jejunal Peyer's patch follicles contain fewer surface IgM^+ B cells, more plasma cells (IgM, IgG1 and IgA), many isotype switched B cells and 10-15 % T cells (Griebel & Hein, 1996).

There are several features that suggest that the ileal Peyer's patches are the major site of production of peripheral B lymphocytes in the sheep. Although massive numbers of B cells are produced in the ileal Peyer's patches only about 5 % of these cells make their way into the peripheral circulation. The remaining 95 % of B cells die *in situ* by apoptosis (Reynolds, 1986). The lymphopoiesis of B cells occurring in the ileal Peyer's patches is analogous to that of T cells in the thymus where there is intense ongoing selection of T lymphocytes. If the ileum is surgically removed early in development, the animal suffers a long-lasting deficiency of B cells in the peripheral circulation (Gerber *et al.*, 1986). The B cells that exit the ileal Peyer's patches migrate to all peripheral tissues. However, unlike lymphocytes from other mucosal sites they do not show any tendency to home back into the gut (Reynolds *et al.*, 1991). Likewise, labelled thoracic duct lymphocytes that are returned to the peripheral blood show very limited localisation to the ileal Peyer's patches. This indicates that the ileal Peyer's patch is not a prominent recirculation pathway for mature lymphocytes (Griebel & Hein, 1996).

Unique features of immunoregulation at mucosal surfaces: The intestinal tract is exposed to a wide array of antigens on a regular basis. The gut mucosal immune system is constantly confronted with the task of discriminating between innocuous and harmful antigens. If the antigen is a harmful pathogen it is essential that an appropriate immune response be mounted against it. However, it is equally important that an identical immune response is not mounted against harmless or potentially beneficial antigens such as food or commensal bacteria.

The mechanisms that help the gut mucosal immune system to discriminate between pathogens and harmless antigens are poorly understood.

The normal physiological response to an orally administered soluble antigen is systemic immunological unresponsiveness. This phenomenon commonly referred to as oral tolerance can be provoked by most inert T cell-dependent antigens, including proteins, peptides and inactivated bacteria or viruses (Weiner, 1997). Evidence also suggests that a similar state of tolerance may be involved in the response of the gut immune system to commensal bacteria (Duchmann *et al.*, 1996). In contrast, live organisms not normally resident in the gut, particulate and T cell-independent antigens stimulate active local and systemic cell mediated and humoral immunity (Mowat & Viney, 1997; Weiner, 1997). It is presumed that any form of antigen which induces oral tolerance cannot induce co-stimulatory activity in antigen presenting cells (Mowat & Viney, 1997).

There is very little consensus among researchers regarding the mechanisms that maintain oral tolerance. Three primary immunological mechanisms have been implicated in oral tolerance: clonal deletion, clonal anergy and the generation of active regulatory mechanisms (Strobel & Mowat, 1998). Clonal deletion is rarely found in peripheral tolerance and has not been described during oral tolerance induction in normal animals. When T cell receptor transgenic mice are fed with unphysiologically large doses of antigens oral tolerance is thought to be induced by clonal deletion (Chen *et al.*, 1995). There is *in vitro* evidence that orally tolerised lymphocytes die by apoptosis. High doses of antigen given to normal mice induce clonal anergy (Whitacre *et al.*, 1991). The active regulatory mechanisms that maintain oral tolerance may include the preferential activation of $CD8^+$ or γδ T cells, the down-regulation of inflammatory Th1 $CD4^+$ T cells by Th2 cells, or the preferential production of inhibitory cytokines such as IL-4, IL-10 or TGF-β (Mowat & Viney, 1997; Strobel & Mowat, 1998). Although investigators have emphasised the importance of different mechanisms, it is very likely that all these mechanisms could operate in the gut and the relative importance of each mechanism might only vary with the antigen involved (Mowat & Viney, 1997).

Developmental aspects of sheep gut immune system: The study of the immune response of the foetus serves two important purposes. The factors involved in the ontogenesis of the immune system are poorly understood. Investigations on the ontogeny of the developing immune system would contribute to an understanding of the fundamental nature of the immune response and its relationship to other physiological systems. Secondly, the mammalian foetus offers an almost unique opportunity for the study of the immune response in an immunologically virgin environment, uncomplicated by the confounding effects that normal animals are subjected to. The foetal lamb has proved to be an excellent subject for this type of study. The placenta of the pregnant sheep does not permit passage of γ-globulins from the ewe to the foetus (Silverstein *et al.*, 1963). Thus, the development of the immune system of the foetal lamb occurs in the absence of extrinsic antigens and maternal antibodies.

A number of investigators have assessed the effect of antigen on the systemic immune response of the foetal lamb. The foetal lamb shows a good response to a wide range of systemically administered antigens (Richardson *et al.*, 1968; Silverstein *et al.*, 1963). In addition Richardson *et al.* (1971) have demonstrated that, after primary immunisation of the foetus, a secondary antigenic stimulus either *in utero* or at birth can elicit antibody levels comparable with those of adult animals. Likewise, it has also been demonstrated that foetal lamb sensitised *in utero* with an antigen can manifest an anamnestic response to the homologous antigen 4-5 months after birth (Tierney *et al.*, 1997). The foetal lamb responds to many antigens with relatively high levels of antibody synthesis, in contrast to newborn lambs, which respond with little or no antibody. The neonatal lamb appears in many ways to be less immunocompetent than the third trimester foetus (Tierney *et al.*, 1997).

Most of the investigations have concentrated on assessing the effect of antigen on the systemic immune response of the foetal lamb. Only a few investigators have focussed their efforts on the effect of antigen on the development of the mucosal immune system. Richardson and Conner (1972) were among the first to investigate the feasibility of stimulating the immune response in foetal lambs by the oral route of sensitisation. Foetal lambs (10 to 75 days prenatally) were exposed to *Brucella* antigen in the amniotic fluid. Serum

agglutinins could be detected in blood collected by cardiac puncture. Although a clear cut response to the antigen could be demonstrated, they could not draw any conclusions regarding the extent of antibody response at different stages of maturation of the foetus because of the small numbers of foetuses and the differences in the time of bleeding. Richardson and Conner (1972) also evaluated the secondary immune response of the foetal lamb to *Brucella* antigen. Primary immunisation of foetal lambs (90 to110 days of gestation) was carried out by the intracardiac route. After 23-37 days, secondary exposure to the same antigen was undertaken using the oral route (antigen was deposited in the amniotic fluid). Seven days after the secondary oral antigenic exposure, high levels of antibody could be detected in the peripheral blood of the foetal lambs. Imprints of the ileum prepared seven days after the oral antigenic stimulation revealed bacterial particles in macrophages-like cells (Richardson & Conner, 1972).

Conner *et al.* (1973) have reported that ovine foetuses vaccinated with an *Escherichia coli* antigen by the oral route can be subsequently protected against otherwise virulent bacterial challenge in postnatal life. The conclusions from their work were based on the observation of a very limited number of animals and, may be questioned.

Husband and McDowell (1975) investigated the immune responses of newborn lambs following prenatal oral immunisation with horse spleen ferritin. Foetal lambs were immunised orally 6-15 days before birth by introducing ferritin into the amniotic fluid. The lambs were sacrificed at birth before colostrum feeding. Blood and intestinal contents were collected while single cell suspensions were prepared from the jejunum and mesenteric lymph node. Specific antibody was detected in the serum and intestinal contents of all antigen-exposed lambs. The antibody activity in the serum was associated with IgM and that in the intestinal contents with IgA and IgM. Relatively high proportions of cells secreting specific antibody were present in the gut tissue of antigen-exposed lambs. In the mesenteric lymph node IgM^+ cells predominated with a small number of IgA^+ cells. In the jejunum approximately equal numbers of IgA and IgM cells secreting specific antibody could be detected (Husband & McDowell, 1975).

Reynolds and Morris (1984) investigated the effect of antigen on the development of the Peyer's patches in foetal lambs. They isolated about half the length of the ileum containing the Peyer's patches from the intestinal tract during the last month of pregnancy. Antigen was injected into this isolated loop and the subsequent development of the Peyer's patches was studied. The injection of either killed *Brucella abortus*, ferritin or maternal colostrum into the lumen of the isolated ileal segment did not cause premature growth of the Peyer's patch follicles nor did it affect their lymphoblast content. In contrast, the injection of antigen into isolated segments evoked the development of germinal centres and plasma cells in the regional mesenteric lymph nodes. Plasma cells also appeared in the lamina propria along the intestinal tract in response to the antigens. The results of Reynolds and Morris (1984) have provided experimental evidence that lymphopoiesis in the ileal Peyer's patches is not dependent on antigen. The Peyer's patches in ileal segments that were not injected with antigen developed at the normal rate before and for the first two weeks after birth. However, after this the growth of the follicles became significantly slower than that of normal Peyer's patches. The follicles in these isolated segments completely disappeared by 3-4 months of age. In marked contrast, the follicles in the normal functional ileum did not undergo involution until around 15 months of age. Likewise, Reynolds and Morris (1984) also observed that premature involution of the Peyer's patches in the isolated segments could be prevented by reconnecting the segment to the functional intestinal tract before three months of age.

General features of parasitic infections: Significantly high proportions of human and animal populations are infected with helminths. Helminths are multicellular pathogens. There are three major groups of helminths namely nematodes, cestodes and trematodes. Although chemotherapy is available for the major helminth diseases, individuals remain highly susceptible to reinfection. The eukaryotic parasites are characterised by their elaborate life cycle in comparison with prokaryotes and viruses. Very often the parasites undergo a succession of developmental stages within the same host. These stages may express stage-specific antigens, which are thought to be extremely beneficial to the parasite in evading or curtailing the effects of any immune response that is generated (Maizels *et al.*, 1999).

A few generalisations can be made about helminthic infections. The most conspicuous of these relates to the high levels of T helper 2 type responses generated. The Th2 type immune response is characterised by high levels of IgE antibodies, eosinophilia and mast cell proliferation. All these effects are cytokine mediated (Finkelman *et al.*, 1997). Very often the Th2 response generated is protective and serves to eliminate or curtail the infection (Mosmann & Sad, 1996). The stereotype Th2 response to gastrointestinal nematode infection has been well documented in mice and humans. Domesticated animals infected with nematodes also showed a tendency for a polarised immune response. However, the classical, highly polarised immune response seen in mice is yet to be reported in domestic animals like sheep.

Helminth parasites exert their pathogenic effects by establishing long-term, chronic infections which often result in pathological damage to the host. It is believed that much of the pathological damage is inflicted by the host's own immune response to the infection rather than by the parasite itself (Maizels *et al.*, 1999).

***Trichostrongylus colubriformis* infection in sheep:** *Trichostrongylus colubriformis* is an important parasitic nematode of sheep, being one of the three gastrointestinal nematode species of major economic importance to the Australian sheep industry. It has a simple lifecycle, with the adult inhabiting the proximal small intestine (chiefly the first three metres) and the eggs being passed in faeces. The eggs hatch on pasture, and the infective third-stage larvae are ingested by the sheep, exsheath in the abomasum and mature in the small intestine. The adults live in mucus-covered tunnels eroded into the surface of intestinal villi, with much of the worm remaining free in the lumen.

Infection with *T. colubriformis* influences the gastrointestinal function of the host by depressing food consumption (Steel *et al.*, 1980) impairing uptake of nutrients (Steel & Symons, 1982) and increasing endogenous nitrogen secretion into the gastrointestinal tract (Poppi *et al.*, 1986). There is also a cost to the host during acquisition of protective immunity, consisting of both physiological (gastrointestinal function) and production (wool growth and live weight gain) costs (Kimambo *et al.*, 1988). Once established, protective immunity appears to impose no detectable metabolic or production cost (Kimambo & MacRae, 1988).

Most adult ruminants exhibit naturally-acquired protective immunity to endemic *Trichostrongylus* spp. However, immunologically naive animals are vulnerable to infection and disease for a period, as protective immunity takes several months to develop. The speed with which immunity develops is influenced by the dose of larvae ingested (Dobson *et al.*, 1990). As protection develops, animals sequentially acquire the ability to reject incoming larvae (after 5-7 weeks of continuous exposure), depress worm fecundity (after 10-12 weeks), and finally to expel adult worms (16-20 weeks later).

Mechanisms of worm rejection: Since *Trichostrongylus colubriformis* infection is solely restricted to the gastrointestinal tract the nematode is relatively isolated from the systemic immune response. In addition, the development of immunity against the worm requires suppression of the active mechanism of oral tolerance which itself can suppress immune responses to proteins ingested in food. A range of protective responses, both innate and acquired, are at the disposal of the infected host to tackle the nematode infection. No single mechanism described to date has been found sufficient or adequate to mediate immunity on its own. Thus, immunity to the nematode is thought to be maintained by several different protective mechanisms acting in concert (Emery *et al.*, 1993). The protective mechanisms include gut peristalsis, altered pH, mucous entrapment, the epithelial barrier, local inflammation, nervous, cellular and humoral responses, and systemic humoral responses.

When adult sheep are fully immune to *T. colubriformis* and have been exposed within the previous 7 weeks ('hyper-immune'), they can mount a 'rapid rejection' response, eliminating incoming *Trichostrongylus* larvae from the entire 15 metres of the small intestine within 2 hours (Wagland *et al.*, 1996). This is thought to be a mucosal mast cell-mediated hypersensitivity response, and is associated with the appearance of large numbers of mucosal mast cells and globule leucocytes, and with local production of mast cell protease. If this hyperimmune state has lapsed, or previous exposure was insufficient to generate this degree of immunity, rejection takes somewhat longer - approximately 5-14 days (McClure *et al.*, 1992). It is then associated with increases in local lymphocytic and humoral immune responses, in addition to the mast cell involvement.

Despite considerable research into the responses occurring at the time of immune rejection of worms, most of these responses should be regarded as no more than associations or correlations at this stage. It is unclear whether the effects observed are 'causal' or 'casual'. One of the approaches used to address the question of which responses are protective has been to observe the effects on elimination of the parasite after the deletion of individual components of the *in vivo* response during primary or secondary exposure to the infection (Emery *et al.*, 1993). One example of the use of this type of approach has been that of McClure *et al.* (1996). They administered monoclonal antibodies against $CD8^+$, $\gamma\delta^+$ T cells and interferon-γ during the induction of the immune response to *Trichostrongylus colubriformis* infection and observed that protection of sheep to the nematode was actually enhanced by this treatment.

Immune response of lambs to *Trichostrongylus colubriformis* infection: The thrust of the present study was characterisation of the immune response of perinatal lambs to *Trichostrongylus colubriformis* infection after having attempted to interfere with the development of the gut mucosal immune system *in utero*. Thus, the subject of immune response of lambs to *Trichostrongylus colubriformis* infection is dealt with in more detail below.

Although lambs are exposed to nematode infections from the moment they begin ingesting herbage they are unable to respond to the infection by the development of a strong resistance until they are 4 to 6 months old (Gibson & Parfitt, 1972). Gibson and Parfitt (1972) investigated the effect of age on the development of resistance by sheep to *Trichostrongylus colubriformis* infection. Based on the faecal egg count, the worm burden at autopsy and the number of eggs per female worm they inferred that sheep over 24 weeks of age are better able to develop resistance to infection than are younger lambs. The resistance to *Trichostrongylus colubriformis* increases as the lambs grow older and is highly developed when they reach 36 weeks of age.

The immune status of lambs can alter the predilection site of *Trichostrongylus colubriformis* within the gut of the infected lamb. Wagland *et al.* (1996) examined the capacity of infective larvae of *Trichostrongylus colubriformis* to establish throughout the small intestine, rather than being restricted to the anterior small intestine in naive and immune lambs (6-9 months

of age). The location of worms was similar in naïve animals given between 10,000 and 80,000 *Trichostrongylus colubriformis* larvae, with 90 % of the worms localized in the proximal three metres of the small intestine. The worms recovered from immune lambs were also found in the first nine metres of the small intestine. However, the worms recovered from immune lambs were significantly relocated caudally, compared with their position in naïve animals, from the first three into the next six metres of the small intestine. Thus development of immunity in lambs was accompanied by a distal 'migration' or 'relocation' of the parasite from the jejunum towards the ileum. Another important observation made by Wagland *et al.* (1996) was that, although the principal site for the establishment of *Trichostrongylus colubriformis* was the proximal three metres of the small intestine, the immune response generated from this location caused eviction of *Trichostrongylus colubriformis* larvae and adult worms from all regions of the small intestine and the caecum.

Studies have revealed that the dietary protein level has an effect on resistance to parasitic infection in young lambs. The development of immunity and resistance to *Trichostrongylus colubriformis* was evaluated in lambs of 8-26 weeks and 33-51 weeks of age, that were fed with two levels of dietary protein (11 % and 20 %) (Kambara *et al.*, 1993). The resistance to challenge infection was ascertained by measuring worm burden, faecal egg count and eggs *in utero* in the nematode. Young lambs which were offered the low protein diet showed a significantly lower resistance to the parasite than older lambs. The development of immunity was measured by an *in vitro* lymphocyte (peripheral blood) blastogenic test to non-specific mitogens and to third stage *Trichostrongylus* larval antigens. Lymphocyte responsiveness to the mitogens and larval antigens was evident only in older lambs on the high protein diet. The lack of this proliferative response in young lambs led Kambara *et al.* (1993) to suggest that parasite antigen-induced activation of the peripheral blood lymphocytes may play only a minor role in the development of resistance to *Trichostrongylus colubriformis* among young lambs.

Two divergent lines of Romney sheep have been selected on the basis of their resistance or susceptibility to gastrointestinal nematodiasis. Pernthaner *et al.* (1995) have reported that the two lines of Romney sheep manifest different

immune responses to *Trichostrongylus colubriformis* infection. Lambs six months old were infected with 5000 infective larvae of *Trichostrongylus* twice weekly for a total of 13 weeks. They observed that, compared to pre-infection levels, the blastogenic activity of unstimulated lymphocytes in lambs of both lines peaked three weeks after infection, and was significantly higher in resistant than in susceptible lambs. Lymphocytes from susceptible lambs responded more strongly to mitogens like conA, PHA and pokeweed mitogen than cells from resistant lambs. The blastogenic activity of LPS-stimulated cultures was significantly higher in cells from resistant compared with susceptible lambs between 3-4 weeks post infection. No significant correlation was observed between the decline in faecal egg count and blastogenic activity. The eosinophil counts in peripheral blood began to increase one week earlier in resistant than in susceptible lambs. No significant correlation between the faecal egg count and eosinophil counts was observed in resistant lambs. In contrast, a significant correlation was found between faecal egg count and eosinophil count in susceptible lambs at some sampling times.

A few investigators have characterised the lymphocyte phenotype in the peripheral blood of *Trichostrongylus colubriformis* infected lambs. Pernthaner *et al.* (1996) infected Romney lambs (7 months old) from both susceptible and resistant lines with 10,000 infective larvae of *Trichostrongylus axei* at weekly interval for 14 weeks. They observed significant increases in the proportions of $CD5^+$, $CD4^+$, $CD8^+$ and $\gamma\delta$ cells in both resistant and susceptible lines of lambs during the first four weeks of infection. After reaching peak levels, the proportion of $CD5^+$, $CD4^+$ and $CD8^+$ cells fell with the rate of decline of $CD5^+$ and $CD4^+$ cells being significantly greater in the resistant line lambs. Susceptible line lambs showed higher proportions of $CD5^+$ and lower proportions of $CD45R^+$ cells than resistant lambs before infection with *Trichostrongylus*.

Kambara and McFarlane (1996) also investigated the changes in the T cell subpopulations of the peripheral blood of Trichostrongylus colubriformis infected lambs. Young lambs (8-26 weeks) and older sheep (31-51 weeks) were infected with a low dose of Trichostrongylus colubriformis larvae for four successive weeks and the changes in the lymphocyte phenotype were monitored. During the course of sensitisation, the older animals exhibited an increase in the percentage

of $CD5^+$, $CD4^+$ and $CD8^+$ T cells. On the contrary, the percentage of $CD5^+$, $CD4^+$ and $CD8^+$ cells decreased in the young lambs. The percentage of γδ T cells was significantly higher in younger lambs compared with older animals.

The antibody response in the peripheral blood following *Trichostrongylus colubriformis* infection of lambs (8 months) has been shown to be predominantly of the IgG1 and IgM isotypes (Douch *et al.*, 1994). The IgG1 response was more persistent than the IgM response. Dexamethasone treatment of lambs curtailed the antibody responses and elevated the faecal egg count in such animals (Douch *et al.*, 1994). A serum IgE response during both primary and challenge infection of young lambs (3-4 months) with *Trichostrongylus colubriformis* has been reported by Shaw *et al.* (1998). During the primary infection, the parasite specific IgE response peaked between 20-27 days post-infection. The parasite specific IgE response during challenge infection was much more rapid with peak levels attained by 7-8 days post-challenge.

The number of reports of investigations of the immune response generated to *Trichostrongylus colubriformis* infection in the gut of young lambs seems to be very limited. McClure *et al.* (1998) attempted to generate immunity against *Trichostrongylus colubriformis* in perinatal lambs (1 month) by vaccination with the viable parasite. Lambs were infected by repeated, continuous low dose (trickle) administration of *Trichostrongylus colubriformis* infective larvae or by truncated infection involving the administration of high doses of viable larvae followed by anthelmintic administration to prevent the maturation of the larvae. These lambs and a previously untreated control group, were subsequently challenged with a single bolus infection of 30,000 larvae and then sacrificed either one or seven weeks later. The worm count after challenge was significantly lower only in the trickle vaccinated lambs. They had a mean protection of 76 % at one week after challenge and 45% by seven weeks post-challenge. The gut histology of the lambs revealed significant differences one week after challenge with the control and truncated infection groups of lambs revealing only a mild hyperaemia. In contrast, lambs exposed to trickle vaccination showed severe inflammation, villous atrophy, mononuclear cell infiltration, hyperaemia and increased numbers of mast cells, globule leukocytes,

and, in some lambs, eosinophils. Immunoperoxidase staining for surface antigen detected similar numbers of mucosal cell types in all the groups except that trickle vaccinated lambs had more $CD1^+$ and γδ cells. At seven weeks after challenge, none of the groups of lambs showed any signs of active inflammation but some sheep in the trickle and truncated infected groups showed signs of jejunal villus atrophy with regenerated epithelium. However, the immunohistology that would be required to further characterise the mucosal cells phenotypes was not done at this time. Mast cells from the gut of lambs immunised by trickle and truncated infection, but not from control sheep, released significant levels of protease *in vitro* when they were stimulated with soluble third stage larval antigen one week after challenge. Mast cells from all groups released protease seven weeks after challenge, but the values for trickle infected sheep were twice those of the control or infection truncated groups. When total and isotype-specific antibody responses in the intestine of lambs were correlated with worm count, a significant correlation was found only with IgM at one week after challenge and IgE at seven weeks after challenge. McClure *et al.* (1998) have suggested that the presence of IgM in previously vaccinated lambs indicated that the lambs were mounting a local antibody response that was still primary in nature and indicative that a long induction period was necessary for the development of protective immunity.

Emery *et al.* (1999) have demonstrated that partial protective immunity to *Trichostrongylus colubriformis* can be induced in neonatal lambs by trickle immunisation with third stage larvae. They believed that the mechanism of rejection of *Trichostrongylus colubriformis* by immune neonatal lambs was not the same as the rapid rejection/ immune exclusion mechanism observed among older sheep. The antibody induced in the mesenteric lymph node was almost exclusively of the IgG1 isotype. Likewise, IL-5 was also induced in the gut of infected lambs (Emery *et al.*, 1999).

The published literature reveals that very little work has been done with regard to the effect of antigen on the development of the gut immune system in the foetal lamb. Likewise, very little in know about the secondary immune response generated in the gut of such prenatal sensitised lambs. The present study was undertaken to investigate these aspects.

The Basis of this present study: Information that is currently available about development of the capacity of the gut immune system to respond to parasitic infection remains insufficient at present to formulate precise hypotheses which lend themselves to experimental testing. To a large extent, this insufficiency reflects the current incomplete understanding of the mechanisms of response of the *mature* gut to parasites and even of the relative importance of process which could be classified as typical *immune* responses in the total response of the gut.

It appears to be inevitable that, when the responsiveness of the gut to parasites has been completely explained, it will be conspicuously multifunctional. The contribution that the gut associated immune system provides as part of the gut response to parasitic infection cannot be assumed *a priori* to be completely beneficial for the animal. Some components of the immune response may be irrelevant to expulsion of the parasite if other mechanisms achieve this more rapidly. Other components, while appearing to be positive from an exclusively immunological perspective, may be decidedly disadvantageous for the animal if, for example, they lead to an excessive inflammatory response in gut tissue.

The evident complexity of the response of the mature gut to parasites and the present inadequate understanding, for instance, which phenomena are causes and which are effects was persuasive in directing the strategy of this project towards examination of the foetal and neonatal gut. This selection runs counter to the "typical" approach of seeking information about mature function before attempting to explain developmental stages of that function. However, there are sound reasons to reverse the normal order when investigating the gut responses to parasites. Whilst exposure of the foetal gut to parasite antigens is an artificial situation, it brings with it the compensating advantage of providing a much simpler situation. Any attempt to examine the consequences, for subsequent challenge with a parasite, of antigenically priming the gut of a mature animal may be confounded by the effects of such uncontrolled influences as gut flora, previous antigenic exposure and the effects of antigenic cross-reactivity and dietary factors.

The specific aim of this project was to compare the responses to infection with *Trichostrongylus colubriformis* in postnatal life of two group of lambs, one of which had been exposed via the gut to *Trichostrongylus colubriformis* larval

antigens in foetal life. Its aim was not to compare events occurring during the response of lambs to *Trichostrongylus colubriformis* infection with the unchallenged gut but to describe the influence of exposure during foetal life on response to later challenge. Selection of the parameters to be compared in the two groups of lambs was determined by earlier reports which had suggested that a specific parameter had been observed, or might be expected, to change in response to parasitic infection. Twenty two parameters were examined in each group of lambs. Eleven of these will be presented in this thesis in detail. These eleven parameters were selected for presentation because they appeared to display differences between the two groups of lambs. Rather than amalgamating the background information relating to each of the parameters for which data will be presented, it is more appropriate to present this in some detail, and to indicate its potential relevance to understanding the development of gut responses, immediately preceding presentation of the data to which it relates.

MATERIALS AND METHODS

Animals:

Pregnant Merino ewes maintained at the **Spring Valley Farm** (Australian National University, Canberra) were used for the present study.

Preparation of *Trichostrongylus colubriformis* infective larval antigen (SPL3 antigen):

The third stage infective larvae of levamisole-susceptible laboratory stocks of *Trichostrongylus colubriformis* were used for the preparation of larval antigen. The soluble larval antigen extract was prepared at 4° C by grinding the larvae in a glass homogeniser. The homogenate was centrifuged at 10,000 g for one hour. The supernatant was collected, filtered through a 0.22 μm filter and stored at –20° C. The protein concentration was estimated using the Biorad method. The *Trichostrongylus colubriformis* larval antigen was used for *in utero* oral antigen exposure of the foetal lambs and for performing ELISA.

Pre-operative preparation and anaesthesia:

Pregnant merino ewes carrying foetuses around 100 days (100 ± 5 days) of gestation were used for the surgical procedure. The ewes were starved of food overnight (6-8 hours) prior to the surgery. **Thiopentone sodium** (Jurox Pty Ltd, NSW, Australia) in a solution at 50 mg/ml, at a dose of 15 mg/kg body weight was injected via the jugular vein to induce general anaesthesia. A size 9.0 cuffed **endotracheal tube** (Rusch, Germany) was introduced into the trachea of the ewe to maintain its airway during surgery. A gas mixture of 1-3 % **halothane** (Fluothane, ICI Australia operations Pty Ltd, Australia) and 100 % oxygen was administered via the endotracheal tube from a **Boyle's anaesthetic apparatus** (British Oxygen Company). Surgery was normally undertaken in the morning and each operation lasted for 25-40 minutes. During the entire operation time the animals were maintained fully anaesthetised by adjusting the halothane : oxygen gas mixture. The site of surgery was prepared by removing the wool covering the abdominal region using an animal clipper with a fine comb blade. The skin was then scrubbed thoroughly with 7.5 % (w/v) **aqueous povidone-iodine** (Betadine Surgical Scrub, ICI Pharmaceutical, VIC, Australia) and then washed with 70 % **ethanol** containing a mixture of 5 % (w/v) **chlorhexidine gluconate** and 4 % (w/v) **isopropyl alcohol** (Hibitane, Faulding Pharmaceuticals, Australia). Sterile

surgical drapes were used to cover all the non-sterile fields leaving only the operation site exposed. All instruments and tubings used for the surgical procedure were sterilised by autoclaving.

Surgical procedure for *in utero* antigen exposure of foetal lambs:

Pregnant ewes were selected at random for the different treatment procedures which are described below. Ewes carrying either a single foetus or twins foetuses were used. All the ewes were subjected to the same operative procedures irrespective of the treatment group. A paramedian incision was carefully made in the abdominal wall to cut through the skin and muscle layers. The gravid uterus was then approached through this incision and the number of foetuses present in the uterus was confirmed by palpation. If there were twins both the foetuses were subjected to the same treatment. A small nick was made on the gravid uterine wall. The head of the foetal lamb was positioned near the nick. A long flexible tube (30 cm long, sterile) containing the antigen was introduced deep into the gut of the foetal lamb. The antigen was forced into the gut lumen by applying positive pressure by means of a syringe attached to the tubing. Each lamb received 500 µg of soluble *Trichostrongylus colubriformis* larval antigen prepared as described above. The total volume of antigen introduced into the gut of the foetal lamb was 500 µl. Those foetal lambs that were treated in this manner were grouped into the '*in utero* antigen-exposed group'. The 'control group' of animals in this study were foetal lambs that were exposed to 500 µl of normal saline by the oral route.

The nick in the uterine wall was closed with a purse string suture and then reinforced with a second layer of Cushings suture. The muscle layer was closed with two layers of continuous sutures while the incision on the skin was sutured together with interrupted suture. In all the layers including the uterine wall 1 **metric silk** (Davis and Greck, Australia) was used as the suture material. **Benzyl penicillin sodium** (CSL Ltd., Vic, Australia) powder was applied to the wound to prevent infection. The animals were allowed to recover in a secluded, open spaced shed. The condition of the ewes was monitored for the next 4-7 days. Ewes recovered uneventfully and were then transferred to the **Spring Valley Farm** (ANU, Canberra). The animals were brought back to the **John Curtin School of Medical Research** (ANU, Canberra) as the time of lambing

approached. Lambing was allowed to occur naturally in secluded lambing sheds. Assistance with lambing was provided only if the ewe was experiencing difficulty. Once the lambs were born they were always confined to a specific shed with mesh flooring throughout the period of study to preclude the possibility of picking up nematode infections. The details of the number of foetuses operated upon are furnished below:

	T. colubriformis larval Ag.	Saline
No: of foetuses exposed	**33**	**17**
No: of lambs finally available for the study	**16**	**16**

Challenge of perinatal lambs with infective third stage larvae of *T colubriformis* :

Third stage infective larvae of levamisole-susceptible laboratory stocks of *Trichostrongylus colubriformis* (McMaster strain) were used for the preparation of larvae for postnatal infection of the lambs. The larval suspension maintained at 4°C was warmed to a temperature of 30°C. The viability of the stock solution was established by examining it under a light microscope. Lambs from both the groups were initially challenged (First challenge) with the infective larvae when they were 4-6 weeks old. Each lamb was orally drenched with 15,000 larvae. The infection was allowed to proceed for one week at which time the second challenge was carried out. Each lamb, irrespective of the group, was again orally drenched with 15,000 infective larvae on that occasion. The infection was allowed to proceed for the next eight weeks. On the 9th week after the first challenge the lambs were sacrificed and samples collected as described later. During the course of the experiment different clinical samples were collected from the lambs at various time points. They are described in their respective sections.

Collection of peripheral blood for flow cytometry:

Twenty ml of whole blood was drawn from the jugular vein of each lamb into a sterile plastic syringe containing 2ml of EDTA (10mg /ml of EDTA in phosphate buffered saline, pH 7.4). The blood was collected on three different occasions during the course of the experiment as described below:

Table-2.1: Details of the Monoclonal antibodies used in the present study

Name of the monoclonal antibody	Molecule specificity / Cells marked	Assays used	Dilution of the monoclonal antibody used	Source	Reference
SBU-T1 (25.91)	CD5: T cells, some B cells	Flow cytometry and Immunohistochemistry	1:10 -Flow cytometry 1:4 -Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Birkebak *et al.*, 1994; Chevallier *et al.*, 1998)
SBU-T8 (24.96)	CD8: T cytotoxic/suppressor cells	Flow cytometry and Immunohistochemistry	1:20 -Flow cytometry 1:5 -Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Maddox *et al.*, 1985b)
SBU-T4 (44.38+44.97)	CD4: T helper cells	Flow cytometry and Immunohistochemistry	1:20 -Flow cytometry 1:5 -Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Maddox *et al.*, 1985b)
CC15	WC1: γδ T cell receptor cells	Flow cytometry and Immunohistochemistry	Neat -Flow cytometry Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(McClure *et al.*, 1995)
SBU-p220	CD45R: B cells, some T cells	Flow cytometry and Immunohistochemistry	1:10 -Flow cytometry 1:5 -Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(MacKay *et al.*, 1987)
SBU-CII	MHC class II	Flow cytometry and Immunohistochemistry	1:20 -Flow cytometry 1:5 -Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Puri *et al.*, 1985)

Table-2.1: Details of the Monoclonal antibodies used in the present study (continued)

Name of the monoclonal antibody	Molecule specificity / Cells marked	Assays used	Dilution of the monoclonal antibody used	Source	Reference
SBU-T6 (20.27)	CD1c: Dendritic cells, thymocytes, monocytes, some B cells	Immunohistochemistry	1:4 Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Mackay *et al.*, 1985; Rhind *et al.*, 1996)
CC-20	CD1b: Dendritic cells, thymocytes	Flow cytometry and Immunohistochemistry	Neat- Flow cytometry Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Dutia & Hopkins, 1991; Hopkins & Dutia, 1991)
CAB IL-1 α (10.82)	Interleukin-1 α	Immunohistochemistry	1:50 Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Egan *et al.*, 1994a)
CAB IL-1 β (3.41)	Interleukin-1 β	Immunohistochemistry	1:50 Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Egan *et al.*, 1994a)
CAB TNF-α (6.09)	Tumor necrosis factor α	Immunohistochemistry	1:50 Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Egan *et al.*, 1994b)
MCA797 (mouse ∝ human TGF-β)	Transforming growth factor-β1	Immunohistochemistry	1:5 Immunohistochemistry	Serotec Ltd., Oxford, England	(Gonnella *et al.*, 1998)

Table-2.1: Details of the Monoclonal antibodies used in the present study (continued)

Name of the monoclonal antibody	Molecule specificity / Cells marked	Assays used	Dilution of the monoclonal antibody used	Source	Reference
∝ IgM	Immunoglobulin M	Flow cytometry, ELISA and Immunohistochemistry	Neat -Flow cytometry Neat- ELISA Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Douch *et al.*, 1994)
∝ IgG1	Immunoglobulin G1	Flow cytometry, ELISA and Immunohistochemistry	Neat -Flow cytometry Neat- ELISA Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Beh, 1987)
∝ IgG2	Immunoglobulin G2	Flow cytometry, ELISA and Immunohistochemistry	Neat -Flow cytometry Neat- ELISA Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Beh, 1987)
∝ IgA	Immunoglobulin A	Flow cytometry, ELISA and Immunohistochemistry	Neat -Flow cytometry Neat- ELISA Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Douch *et al.*, 1994)
∝ IgE	Immunoglobulin E	Flow cytometry, ELISA and Immunohistochemistry	Neat -Flow cytometry Neat- ELISA Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Shaw *et al.*, 1998; Shaw *et al.*, 1997)
DU12-24-3	Eosinophils	Flow cytometry and Immunohistochemistry	Neat -Flow cytometry Neat-Immunohistochemistry	Dr. Susan McClure, McMaster Lab., CSIRO, NSW, Australia	(Hein, 1999)

Table-2.1: Details of the Monoclonal antibodies used in the present study (continued)

Name of the monoclonal antibody	Molecule specificity / Cells marked	Assays used	Dilution of the monoclonal antibody used	Source	Reference
M 0873- Monoclonal mouse ∝ human neuron specific enolase	γ γ Enolase: neurons, tumor cells, lymphocytes	Immunohistochemistry	1:5 Immunohistochemistry	Dako, Denmark	(Stewart *et al.*, 1995)
PCNA-purified mouse ∝ human monoclonal antibody	Proliferating cell nuclear antigen	Immunohistochemistry	1:5 Immunohistochemistry	PharMingen International, USA	(Hailata *et al.*, 1995)
MCA 920- Mouse ∝ ovine CD14 (Clone VPM 65)	CD14: Ovine macrophages	Immunohistochemistry	1:4 Immunohistochemistry	Serotec Ltd., Oxford, England	(Gupta *et al.*, 1996)
SBU-LCA	CD45: Leukocyte common antigen	Flow cytometry and Immunohistochemistry	1:5 -Flow cytometry 1:4 -Immunohistochemistry	Centre for Animal Biotechnology, University of Melbourne, Vic, Australia	(Maddox *et al.*, 1985a)

i) Before challenge with *Trichostrongylus colubriformis* larvae- when the lambs were between 3-5 weeks old.

ii) Three weeks (19-22 days) after the first *Trichostrongylus colubriformis* larval challenge.

iii) Eight weeks (54-58 days) after the first *Trichostrongylus colubriformis* larval challenge.

Preparation of peripheral leukocytes for flow cytometry:

The peripheral blood collected for flow cytometry was further processed as described below:

i) The whole blood sample was spun at 1200 g for 20 minutes. The buffy coat that separated out was carefully aspirated using a siliconised Pasteur pipette into a plastic tube.

ii) The buffy coat suspension was mixed with a lysis buffer to lyse the contaminant red blood cells present. The lysis buffer used was a solution of 0.83 % **ammonium chloride** (Ajax Chemicals Ltd., Australia) and 0.17M Tris buffer (Tris [hydroxymethyl]-aminomethane, Sigma Chemical Co., Mo, USA) mixed in a ratio of 9:1. The lysis buffer was pre-warmed to 30° C before it was mixed with the buffy coat suspension at a ratio of 4:1. To ensure complete lysis of the erythrocytes the mixture was mixed gently for 1-2 minutes.

iii) The cell suspension obtained was centrifuged at 1200x g for 10 minutes. The cell pellet obtained (ranging in volume of 400-700 µl) was washed twice with 10 ml of **phosphate buffered saline** containing 2% **Bovine serum albumin** (Sigma Chemical Co, Mo, USA) and 2mg/ml of **EDTA** (PBS/BSA/EDTA buffer).

iv) After the final wash the cell pellet was suspended in PBS/BSA/EDTA buffer so that about 50 µl of the cell suspension contained approximately 2 x 10^6 cells.

Monoclonal antibody staining of peripheral leukocytes for flow cytometry:

i) Fifty µl of the primary monoclonal antibody was added to each well (V shaped bottom) of the **96 well plate** (Corning Costar Corp., USA). This amount of monoclonal antibody was sufficient for 1-2 x 10^6 cells/ well.

The details of the primary antibody used are furnished in Table-2.1. The antibody negative control used was normal mouse serum at a dilution of 1:500.

ii) Approximately 2 x 10^6 cells (ie about 50 µl of the cell suspension) were added to each well. The plate was sealed with a plastic sealer and incubated at 4°C for 30 minutes. Throughout the period of incubation the plate was gently shaken by means of a plate shaker.

iii) After the incubation period the plate was centrifuged in a centrifuge (Model TJ-6, Beckman Instruments Inc, CA, USA) at 250x g for 3-4 minutes. It was then washed and re-suspended three times in 150 µl of PBS/BSA/EDTA buffer.

iv) The second antibody added was 50 µl of 1:400 **Anti-mouse immunoglobulin R-phycoerythrin conjugated** (Silenus Laboratories, Australia). The second antibody was mixed with the cells suspension and the plate was sealed with a plastic sealer and incubated at 4°C for 30 minutes. Throughout the period of incubation the plate was gently shaken by means of a plate shaker.

v) After the incubation period step three was repeated. After the final wash the cells were suspended in 3 % **formaldehyde** (Methanol free, Ultrapure, Polysciences Inc, USA) for fixing. The plates were sealed, covered in aluminium foil and stored at 4°C. The fixed cells were always analysed within three days of staining.

Flow cytometry study:

All the flow cytometry studies were conducted at the John Curtin School of Medical Research using the **FACScan Flow cytometer** (Fluorescence Activated Cell Analyser, Becton Dickinson, San Jose, CA, USA). The FACScan equipped with an argon laser operating at 15mW power and 488 nm wavelength was used to excite phycoerythrin. The standard configuration for spectral discrimination of fluorescence emission contained three ranges of 530/30, 575/20 and 670 LP nm for FL1, FL2 and FL3 detectors respectively.

The scatter pattern of antibody negative control cells was first analysed and used as the reference control for each sample. Initially, the scatter pattern exhibited by cells stained with the different primary antibodies stained cells were

analysed before the gate was applied. The samples were then gated on both forward light scatter and 90° light scatter to exclude cell debris and clumps. The number of cells counted ranged from 50,000-75,000 events for each sample.

Acquired data were saved and, subsequently analysed by the computer package CellQuest Ver 3.1f (Becton Dickinson, San Jose, CA, USA). All the dot plots and histograms used in the analysis step were similar to ones used in the acquisition step. The values obtained were expressed as percentages.

Collection of serum from peripheral blood for assessing the antibody response:

Blood was collected from the jugular vein without any anti-coagulant for the separation of serum. Ten millilitre of blood was collected from each lamb. After the blood clotted it was spun down to separate the serum. The serum samples were aliquoted into **Nunc CryoTube vials** (Nalgene Nunc International, Denmark) and stored at -70°C. Serum was collected from lambs on three different occasions during the course of the experiment. They were as shown below:

i) Before challenge with *Trichostrongylus colubriformis* infective larvae- when the lambs were between 4-6 weeks old. The blood was usually collected 2-3 days before the first challenge with the larvae.

ii) Four weeks after the first *Trichostrongylus colubriformis* larval challenge- between 26-30 days after the first larval challenge.

iii) Nine weeks after first *Trichostrongylus colubriformis* larval challenge- the serum sample was collected just before the lambs were sacrificed.

Estimation of the total antibody titre against *Trichostrongylus colubriformis* infective larval antigen in serum by ELISA:

The protocol adopted for the assay was as described below:

i) Each well (Flat bottom) of the 96 well plate was coated with 0.5 μg of ***Trichostrongylus colubriformis* larval antigen** (prepared as described previously) dissolved in carbonate bicarbonate buffer pH 9.6. One hundred μl of the antigen solution was used to coat each well. The sealed plate was incubated at room temperature overnight.

ii) The next morning the antigen solution was flicked out and the wells were blocked with 2 % **Coffee-mate** (Carnation, NSW Australia)

dissolved in **phosphate buffered saline** pH 7.2 (Coffee-mate PBS). Each plate was blocked with 150 µl/well of the blocking buffer for 90-120 minutes at room temperature.

iii) Serial ten fold dilutions of the neat serum samples were prepared in the Coffee-mate PBS. The dilutions were carried out so that the final volume of the sample that remained in each well was 100 µl. The plate was incubated at room temperature for 90 minutes.

iv) The serum dilutions were flicked out after the incubation period was over. The plates were washed 5 times in a plate washer using Coffee-mate PBS.

v) One hundred µl of the **anti-sheep IgG (whole molecule), alkaline phosphatase conjugate** (Sigma Immunochemicals, USA) diluted in Coffee-mate PBS at a concentration of 1:20,000 was added to each well. The plate was incubated at room temperature for 60 minutes.

vi) After the incubation period was over the secondary antibody was flicked out. Washing was done as in step iv.

vii) One hundred µl of substrate was added to each well. The substrate used **was 4-Nitrophenyl phosphate** (Boehringer Mannheim, Germany) dissolved in **diethanolamine buffer** at a concentration of 2 mg/ml. (Diethanolamine buffer was prepared by mixing 115 ml of 1M Diethanolamine and 5 ml of 100 mM of Magnesium chloride and making up the volume to one litre with deionised water. The pH of the buffer was adjusted to 9.8 with concentrated hydrochloric acid). The absorbance was measured at 405 nm when the optimal intensity of colour had developed in the plate.

viii) The antibody titre in the sample was estimated using the 'Titrecal" software (Tremain, 1993). The program uses a generalised four parameter logistic curve fit to calculate the titre from the ELISA data. The cut off point for calculation of end-point titre was taken as twice the absorbance reading of the diluent system (Coffee-mate PBS).

Estimation of isotype specific antibody titre against *Trichostrongylus colubriformis* infective larval antigen in serum by ELISA:

ELISA was used to detect the parasite specific antibody isotypes namely IgM, IgG1, IgG2, IgA and IgE in the serum. The protocol used for the assay was as described below.

i) Each well (Flat bottom) of the 96 well plate was coated with 0.5 µg of ***Trichostrongylus colubriformis*** **larval antigen** (prepared as described previously) dissolved in carbonate bicarbonate buffer pH 9.6. One hundred µl of the antigen solution was used to coat each well. The sealed plate was incubated at room temperature overnight.

ii) The next morning the antigen solution was flicked out and the wells were blocked with 2 % **Coffee-mate** dissolved in **phosphate buffered saline** pH 7.2 (Coffee-mate PBS). Each plate was blocked with 150 µl/well of the blocking buffer for 90-120 minutes at room temperature.

iii) Serial five fold dilutions of the neat serum samples were prepared in Coffee-mate PBS. The dilutions were carried out so that the final volume of the sample that remained in each well was 50 µl. Incubation was carried out at room temperature for 90 minutes.

iv) The serum dilutions were flicked out after the incubation period was over. The plates were washed 5 times in a plate washer using Coffee-mate PBS.

v) Fifty µl of the respective **anti-sheep isotype monoclonal antibodies** (prepared in mice) was added to each well. The plate was incubated at room temperature for 90 minutes. (The anti-sheep isotype monoclonal antibodies used were the same as those used for flow cytometry and immunohistochemistry. All of the five monoclonal antibodies were used neat. The details of each anti-isotype monoclonal antibody used in the present study are described in Table-2.1).

vi) After the incubation period the anti-isotype antibody was flicked out. Washing was done as in step iv.

vii) Hundred µl of **anti-mouse IgG (whole molecule) alkaline phosphatase conjugate** (Sigma Immunochemical, USA) diluted in Coffemate PBS at a concentration of 1:5000 was added to each well. The plate was incubated at room temperature for 60 minutes.

viii) After the incubation period was over the anti-mouse IgG alkaline phosphatase conjugate was flicked out. Washing was done as in step iv.

ix) Hundred µl of substrate was added to each well. The substrate used **was 4-Nitrophenyl phosphate** (Boehringer Mannheim) dissolved in **diethanolamine buffer** at a concentration of 2 mg/ml. (Diethanolamine buffer was prepared by mixing 115 ml of 1M Diethanolamine and 5 ml of 100 mM of Magnesium chloride and making up the volume to one litre with deionised water. The pH of the buffer was adjusted to 9.8 with concentrated hydrochloric acid). The absorbance was measured at 405 nm when the optimal intensity of colour had developed in the plate.

x) The antibody titre in the sample was estimated using the 'Titrecal" software (Tremain, 1993). The program uses a generalised four parameter logistic curve fit to calculate the titre from the ELISA data. The cut off point for calculation of end-point titre was taken as twice the abosrbance reading of the diluent system (Coffee-mate PBS).

Faecal egg count :

Faecal samples were collected from the lambs at different times during the course of experiment:

i) One to two days before the lambs were first challenged with the *Trichostrongylus colubriformis* infective larvae. This was done to confirm that the lambs were negative for nematode infections.

ii) Three weeks after the first challenge with the infective larvae of *Trichostrongylus colubriformis* and subsequently once every week thereafter till they were sacrificed.

A total of six samples per lamb were available for performing the faecal egg count. The faecal egg count was performed by the McMaster technique (Whitlock, 1948). Two grams of faeces was soaked in water overnight. The next morning the soaked faecal pellets were mixed and suspended in saturated sodium chloride solution. The suspension was sampled and counted in the Whitlock chamber. Results were expressed as eggs per gram of faeces.

Sacrifice of Lambs:

Lambs from *in utero* antigen-exposed and control groups were sacrificed towards the end of the 9th weeks after the first *Trichostrongylus colubriformis* larval

challenge. The lambs were euthanised by administering **10 ml Valabarb** (Pentobarbitone sodium 300 mg/ml, Jurox, NSW, Australia) intravenously by the jugular vein. The gross lesions if any in the gastrointestinal tact were noted. The following tissue samples were collected:

i) Jejunum : The jejunal sample was collected between 1-2 metres from the pylorus. This tissue was collected because the predilection site of *Trichostrongylus colubriformis* is the anterior portion of the small intestine.

ii) Mesenteric lymph node (MLN): The MLN draining the jejunal region of the gastrointestinal tract was collected. This tissue was collected because the cells involved in the immune response to the nematode are thought to actively traffic through the lymph node.

iii) Ileum containing the Ileal Peyer's patch (IPP): The IPP is thought to act as a primary lymphoid organ in sheep and it was desirable to investigate if any changes were induced in this tissue during the course of study.

The tissue samples collected for immunohistology were placed **in Nunc CryoTube vials** (Nalgene Nunc International, Denmark) containing **Tissue-Tek OCT compound** (Sakura Finetek, USA Inc., CA, USA) and plunged into liquid nitrogen for rapid freezing. Once the samples were frozen the vials were stored at -70° C. The tissue samples collected for antibody assay were collected in cryo-vials without OCT. The vials were then rapidly frozen by plunging them into liquid nitrogen. Once the samples were frozen the vials were stored at -70° C.

Cryosectioning of tissue samples for immunohistochemistry:

The unfixed tissues were sectioned at -20°C after embedding them in **Tissue-Tek OCT compound** (Sakura Finetek, USA Inc., CA, USA). Transverse sections were cut 8 μm thick and then transferred onto gelatin pre-coated slides (Gelatin 0.625 g and Chromium potassium sulphate 0.063 g were dissolved in 250 ml of distilled water. The solution was warmed to dissolve the gelatin. Slides were dipped in the solution for 2-4 minutes till a uniform film of the solution stayed on the slide. The slides were then air-dried). Sections were cut serially for the entire range of markers used. The slides were air-dried, wrapped in aluminium foil and stored at - 70°C.

Single colour immunoperoxidase staining of frozen tissue sections:

The details of the streptavidin immunoperoxidase staining technique used in the present study is given below:

i) The slides were allowed to thaw to room temp before the start of staining. Once fully thawed the slides were labeled. Using a **Pap Pen** (Zymed Laboratories, Inc., CA, USA) a water repellent circle was drawn around the section.

ii) The slides were fixed in chilled **Formol calcium** (4°C) for five minutes. (Formol calcium was prepared by mixing 23 ml of Formaldehyde (35%) and 2 grams of Calcium chloride.$2H_20$ and making up the volume to 200 ml with distilled water. The solution was chilled to 4°C and the pH adjusted to 7.2-7.4 with dilute sodium hydroxide. The solution was stored in the dark and prepared every month for the study).

iii) After the slides were fixed for five minutes the fixative was decanted into the container for re-use. The slides were then rinsed in distilled water and left in a jar of distilled water for five minutes. The distilled water was changed twice during the five minutes period.

iv) Endogenous peroxidase activity in the tissue was quenched by treating the sections with **Methanol peroxide** for five minutes. (Methanol peroxide was prepared freshly before use by mixing 6ml of hydrogen peroxide (30 %) with 94 ml of methanol (99.4 % pure)).

v) At the end of this five minutes the methanol peroxide was decanted. The slides were rinsed in phosphate buffered saline, pH 7.2 (PBS). The slides were then left in a jar of PBS for five minutes. The PBS in the jar was changed twice during the five minutes period.

vi) At the end of the wash the PBS was poured out and the sections were blocked with **0.5 % Bovine serum albumin** (Sigma chemical, Mo, USA) **in PBS containing one drop of normal sheep** serum for every 3 ml of the blocking solution. Slides that were stained for immunoglobulins (IgM, IgG1, IgG2, IgA and IgE) were blocked with 0.5 % BSA solution without any serum. The slides were blocked at room temperature for 20 minutes.

vii) The blocking solution was poured off at the end of twenty minutes and the primary antibody was added. The details of the primary antibody including the dilutions used are as described in the section on primary antibodies (Table-2.1). The slides were incubated in a moist chamber at room temperature for one hour.

viii) At the end of the incubation the primary antibody was discarded. The slides were rinsed in PBS and left in a jar of PBS for five minutes. The PBS in the jar was changed twice during the five minutes period.

ix) **Anti-Mouse Ig-Biotin** [Affinity-purified sheep IgG to mouse immunoglobulins conjugated with D-biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester] (Boehringer Mannheim, Germany) diluted 1: 500 in 0.25% bovine serum albumin in PBS was used as the secondary antibody. Slides were incubated with the secondary antibody in a moist chamber at room temperature for one hour.

x) At the end of the incubation the secondary antibody was discarded. The slides were rinsed in PBS and washed as in step viii.

xi) The staining was enhanced by means of the **Vectastain Elite ABC reagent** (Vector Laboratories, Inc., CA, USA). The slides were incubated with the ABC reagent for 30 minutes at room temperature. (The ABC reagent was prepared using the **Vectastain Elite ABC kit** by mixing one drop of reagent A and one drop of reagent B in 2.5 ml of PBS and mixing well. This solution was always prepared 30 minutes before use.)

xii) At the end of the incubation the ABC reagent was tipped out. The slides were rinsed in PBS and washed as described in step viii.

xiii) The sections were then treated with the chromogen solution prepared using the **DAB Reagent Kit** (Kirkegaard and Perry Lab, Inc. MD, USA). The chromogens solution was incubated on the slides, at room temperature for a few minutes (3-8 minutes) till adequate colour developed. (The DAB solution was prepared freshly each time using the kit. Three drops of Tris buffer were mixed with 5ml of distilled water. To this solution 2 drops of DAB were added, mixed well and then two drops of peroxide were added and mixed well).

xiv) The DAB solution was poured into a jar of water containing hydrogen peroxide. The slides were rinsed with distilled water and then left in a jar of distilled water for five minutes. The distilled water was changed twice during this period.

xv) Counter-staining of the slides was done with **Harris haematoxylin** (BDH, Vic, Australia) for 25 seconds. After this, the slides were immediately washed in distilled water and air dried. (Harris haematoxylin solution was prepared by mixing 10 ml of haematoxylin with 40 ml of distilled water alkalinised with 3 drops of 1 M sodium hydroxide).

xvi) Slides were mounted with a coverslip using **Ultramount with colourfast** (Fronine Pty Ltd, NSW, Australia).

Image analysis of immunoperoxidase stained sections:

The stained jejunal and mesenteric lymph node (MLN) sections were examined in a **Zeiss Axioskop microscope** (Carl Zeiss, Oberkochen, Germany) at a frame width of 1.15 mm (higher magnification). The ileal Peyer's patch (IPP) was examined under a lower magnification at a frame width of 1.8 mm. These frame width were used so that the entire depth of the mucosa could be projected onto the 17" computer monitor. The sections were always oriented so that the mucosal epithelium lay parallel to the top of the screen and the muscularis mucosae was oriented parallel to the base of the screen. Image analysis was carried out on the entire depth of the jejunum and IPP mucosa omitting the muscularis mucosae. The medullary region of the mesenteric lymph node was image analysed after staining with the different monoclonal antibodies. In the case of IL-1 β marker, the cortical region of the mesenteric lymph node was also analysed in addition to the medullary region.

The magnified image formed in the microscope was captured by a **Sony 3 CCD colour video camera** (Sony Corporation, Japan). The image captured by the CCD camera was grabbed by the **QuickImage 24 video frame grabber** (Mass Microsystems, CA, USA) and displayed on the 17'' computer monitor as a grey scale image of 640 x 480 dots per inch resolution. The image was analysed using the **NIH Image** software (Ver 1.61, National Institute of Health, USA). The total area on which the image analysis was performed was held constant for

all the jejunal and MLN samples. Since the framed width used for the IPP was different the total area on which image analysis was done was different from that of the jejunum and MLN. This area was kept constant for all the IPP analysed. The total area stained by the primary antibody was estimated by density slicing of the image. The threshold was varied for the different samples so that the entire positive area stained by the antibody could be detected. This was always done by simultaneous comparison with the image in the microscope. The total area stained by the antibody was expressed in sq.μ.

Staining for Goblet cells and Mast cells:

The staining method developed by Kermanizadeh *et al.* (1995) and modified by Winter *et al.* (1995) was used to stain goblet cells and mast cells in the jejunum.

i) Samples of the jejunum one cm long were fixed in **MT fixative** for 24 hours. (MT fixative was prepared by mixing equal volumes of A: 5 g of mercuric chloride (BDH, Vic, Australia) dissolved in 100 ml distilled water and B: 20 g tannic acid (Sigma Chemicals, Mo, USA) dissolved in 100 ml distilled water and then filtered. Solution A and B were kept for a maximum of one week).

ii) The fixed tissue was embedded in paraffin wax. Sections were cut at 5 μm thickness.

iii) The sections were deparaffinated and rehydrated in graded alcohol: absolute/ 90% alcohol for 2.5 minutes each. The sections were then immersed in 0.5% iodine in 80% ethanol for 3-5 minutes.

iv) The slides were then transferred immediately into 3% sodium thiosulphate in distilled water for 3-5 minutesand then rinsed in running water for 1-2 minutes.

v) The slides were immediately transferred into **1% Astra blue** (BDH, Gurr) (adjusted to pH 0.3 with hydrochloric acid) for 30 minutes.

vi) The slides were rinsed in tap water until the slide was clear and extra stain was removed. Washed the slides in ammonia water for one minute. Rinsed in tap water once again.

vii) The slides were immediately dipped into **Chromotrope 2R** (BDH, Gurr) for 30 minutes. (Chromotrope 2R was prepared by adding one

gram phenol to a flask and melting it gently on a warm water bath. Then 0.5 g chromotrope powder was added and thoroughly mixed to form a slurry. To this slurry 10 ml of absolute ethanol was added and the mixing continued. Finally 100 ml of distilled water was added to this and the solution filtered through Whatmans No.1 filter paper)

viii) The slides were washed in tap water. The sections were quickly dehydrated for not more than 30 seconds each in 30%, 50%, 70%, 90% and absolute ethanol. The slides were blotted dry and immersed in xylene for 3 minutes. The slides were finally mounted in DPX.

Counting of Goblet cells and Mast cells:

The mast cells stained by the above technique were small cells with granular cytoplasm and dark blue in colour. The goblet cells tended to be bigger with clear cytoplasm and stained a pale light blue colour. The number of goblet and mast cells in the jejunum were quantified by counting the number of cells present in each microscopic field (400X). The number of cells in four different microscopic fields were counted for each sample. The mean number of cells per microscopic field was calculated and the results were expressed as number of cells per microscopic field.

Estimation of the total antibody titre against *Trichostrongylus colubriformis* infective larval antigen in jejunum and mesenteric lymph node by ELISA:

The jejunum and MLN tissue were homogenised in chilled PBS pH 7.2 (4°C). The samples were then diluted 1:10 in PBS and used as the starting sample for performing the ELISA. Four fold dilutions of the tissue homogenate were made in PBS Coffee-mate for the assay. The remaining protocol was exactly identical to the ELISA protocol used to estimate the total antibody titre against *Trichostrongylus colubriformis* infective larval antigen in the serum.

Estimation of isotype specific antibody titre against *Trichostrongylus colubriformis* infective larval antigen in the jejunum and MLN by ELISA:

ELISA was used to detect the parasite specific antibody isotypes namely IgM, IgG1, IgG2, IgA and IgE in the jejunum and MLN. The two tissues were homogenised and diluted as described in the section above. Samples that had been diluted 1:10 in PBS were used as the starting sample for performing the ELISA. Four fold dilutions of the tissue homogenate were made in PBS Coffee-

mate for the assay. The remaining protocol was exactly identical to that used for the estimation of isotype specific antibody titre against *Trichostrongylus colubriformis* infective larval antigen in the serum by ELISA.

Statistical Analysis: All statistical analyses for the present study were carried out using **MINITAB release 12** statistical software package (Minitab Inc, USA). The various analyses performed included Analysis of Variance, Chi-square analysis and Pearson correlation. A p-value of less than or equal to 0.05 was considered statistically significant.

CD4

INTRODUCTION

The CD4 molecule has been shown in humans and mice to be a single chain transmembrane glycoprotein which interacts with MHC II molecules (Doyle & Strominger, 1987). T cells expressing CD4 have a restricted capacity for recognition of antigen in association with MHC II (Marrack *et al.*, 1983) and have mainly helper / inducer functions (Swain, 1983).

$CD4^+$ helper T cells from mice and humans have been categorised, based on their function, into Th1 and Th2 subpopulations. Mouse Th1 cells produce IL-2, interferon γ and lymphotoxin β whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th1 cells are involved in cell mediated inflammatory reactions (Mosmann & Sad, 1996; Wood & Seow, 1996). Several Th1 cytokines activate cytotoxic and inflammatory functions and delayed type hypersensitivity reactions. Th2 cytokines encourage antibody production, particularly IgE responses, and enhance eosinophil proliferation and function (Mosmann & Sad, 1996; Wood & Seow, 1996). It is a commonly held assumption that helminthic infections lead to a dramatic induction of the Th2 lymphocyte subset (Allen & Maizels, 1996). Recovery from parasitic infections is generally thought to be favoured by a Th2 response (Mosmann & Sad, 1996).

Although there are many well-documented examples of typical Th1 and Th2 responses, these are not the only cytokine patterns possible. T cells expressing cytokines of both patterns have been called Th0 cells while those producing high amounts of TGF-β have been termed Th3. Additional phenotypes have also been described on the basis of *in vitro* studies (Mosmann & Sad, 1996).

An important issue that is yet to be resolved is whether polarisation of T helper cells into Th1 and Th2 also occurs in species other than mice (Wood & Seow, 1996). Wood and Seow (1996) have reported that in the bovine species, the majority of Th cell clones from parasitic infections such as *Babesia* and *Fasciola* could not be classified either as Th1 or Th2. Brown *et al.* (1998) have observed that the majority of over 60 parasite antigen-specific bovines Th cells clones coexpress IL-4 and interferon γ. Furthermore, IL-2 and IL-10 expression was not restricted to interferon γ or IL-4 producing cells, respectively. Thus a view point that seems to be emerging is that clear cut Th1 and Th2 responses,

per se, are not typically representative of the immune response to most pathogens (Brown *et al.*, 1998).

Distribution of $CD4^+$ lymphocytes in the gastrointestinal tract: The distribution of $CD4^+$ lymphocytes in the gastrointestinal tract depends upon numerous factors such as age, species, anatomical region of the gut and the presence or absence of infection in the gut.

Lamina propria T cells have a CD4: CD8 ratio similar to that seen in the peripheral organs although the functions of these cells are still poorly understood. Virtually all lamina propria T cells in humans and rodents, including $CD4^+$ lymphocytes, express the phenotype of memory/ activated T cells (Mowat & Viney, 1997). Large proportions of the CD4+ T cells actively secrete cytokines *in situ*. It seems that there may be no inherent bias towards one or other Th subset in the lamina propria, although Th2 cytokines may predominate in the absence of inflammation (Mowat & Viney, 1997).

At around 68 days of gestation of the fetal lamb, a few scattered $CD4^+$ lymphocytes are evident in the ileum. Subsequently there is a gradual increase in the number of these T cells. The $CD4^+$ cells tend to accumulate along the base of the crypts and in the villi. By 115 days of gestation, $CD4^+$ cells increases in number to position themselves around the periphery of the follicles. Scattered $CD4^+$ cells can also be seen in the interfollicular region and in the lamina propria of the villi. Beyond 115 days of gestation, $CD4^+$ reactivity and anatomical distribution in the ileum are essentially the same as those of the postnatal lamb (Press *et al.*, 1992).

After birth of the lamb, a rapid increase in the number of $CD4^+$ lymphocytes occurs in the lamina propria of the duodenum. Just eight days after birth, lambs have 40% of the adult complement of $CD4^+$ lymphocytes. At 9 weeks of age, lambs have not attained the adult number of $CD4^+$ cells although they have attained around 70% of this. Lambs acquire adult levels of $CD4^+$ lymphocytes only 5 months after birth (Gorrell *et al.*, 1988b).

In adult ewes, $CD4^+$ T cells comprise the major lymphocyte subset in the forestomach mucosa. $CD4^+$ T lymphocytes account for 1.5% of the total area in image analysis studies (Josefsen & Landsverk, 1996).

Hopkins (1991) has reported that, in the lymph nodes of sheep, $CD4^+$ lymphocytes are found mainly in the paracortex, with a few cells in the follicles and the medullary region. The proportion of $CD4^+$ cells among the peripheral blood lymphocytes ranges from 25-40%.

Hein *et al.* (1989) analysed the lymphocyte composition of the ileal Peyer's patches and jejunal Peyer's patches of five lambs, ranging in age from 5 days to 16 weeks. Immunohistochemical staining of tissue sections revealed that $CD4^+$ lymphocytes were localized to the interfollicular region. FACS analysis showed that <1 % of the lymphocytes in the IPP were positive for CD4. In contrast, 14 % of the cells in the JPP were $CD4^+$ lymphocytes. The extreme paucity of $CD4^+$ lymphocytes in the IPP prompted Hein *et al.* (1989) to suggest that B cell maturation in this tissue is unlikely to be influenced significantly by T cell interactions.

Aleksandersen *et al.* (1990) found that the large intestine Peyer's patches and the JPP of young lambs (52-58 days of old) are almost identical in terms of their $CD4^+$ lymphocyte composition. Between 20-23% of the total lymphocyte population were positive for CD4. The prominence of the T cell component in these tissues suggests that they function as secondary lymphoid tissue. In contrast, $CD4^+$ lymphocytes were scarce in the IPP with <1 % of cells staining with this marker. This gives an indication that differentiation of B lymphocytes in the IPP follicle does not require the participation of T cells.

$CD4^+$ lymphocytes and immunity against gastrointestinal nematodes:
Protective immunity against gastrointestinal nematode parasites is $CD4^+$ T cell dependent. Administration of anti-CD4, but not anti-$CD8^+$, monoclonal antibody prevents expulsion of *Nippostrongylus brasiliensis* and promotes parasite egg production for the duration of the monoclonal antibody treatment (Finkelman *et al.*, 1997). $CD4^+$ T cells have also been shown to play a vital role in protective immunity of immune animals. In mice immune to *Heligmosomoides polygyrus,* reinfection results in an infection that is milder than the primary one, with reduced worm survival and little egg production by surviving worms. Treatment with anti-CD4 monoclonal antibody at the time of challenge infection completely blocks this host immunity (Urban *et al.*, 1991).

Among *Haemonchus contortus* resistant breeds of sheep, the resistance of immune animals is markedly influenced by $CD4^+$ lymphocytes. Gill *et al.* (1993) administered anti-CD4 monoclonal antibody to immune lambs and completely abrogated their resistance to reinfection. The depletion of $CD4^+$ T cells resulted in a significant reduction of mucosal mast cell, globule leucocyte and tissue eosinophil populations. Likewise, their anti-parasitic antibody responses were also markedly inhibited. Karanu *et al.* (1997) found that $CD4^+$ T lymphocyte depletion only partially abrogated immunity induced by parasitic gut antigen immunisation in sheep and goats challenged with *Haemonchus contortus*.

There are strong indications that $CD4^+$ lymphocytes might play an important role in the immunity of sheep to *Trichostrongylus colubriformis* infection. Among immune sheep, the rejection of the parasite from the gut occurs at two or more points of time. The second phase of rejection of *Trichostrongylus* from the gut occurs between five and 10 days post exposure. During this period the lamina propria of the jejunum has an increased population of $CD4^+$ lymphocytes (McClure *et al.*, 1992).

Kambara and McFarlane (1996) sensitised lambs of two age groups with repeated low doses of *Trichostrongylus colubriformis* larvae. Among older animals (31-51 weeks), this produced an increase in the number of peripheral $CD4^+$ lymphocytes. In contrast, among younger animals (8-26 weeks) there was a decrease in the number of lymphocytes staining for CD4 in the peripheral circulation. The older animals also had significantly higher numbers of $CD4^+$ cells in the efferent mesenteric lymph in comparison with the younger lambs. Kambara and McFarlane (1996) have suggested that the unresponsiveness of the younger animals to the parasite may have been a reflection of the inadequate number or functional incapability of the $CD4^+$ lymphocytes, compared with older animals.

Gorrell *et al.* (1988b) sensitised sheep with 10,000 *Trichostrongylus* larvae/week for 7 weeks and subsequently challenged them with different doses of the same parasitic larvae. In all the infected sheep, $CD4^+$ lymphocytes in the lamina propria of the duodenum were concentrated towards the apex of the villi. Challenge with 20,000 or fewer larvae did not increase the number of $CD4^+$

Table- 3.1: Total positive area – $CD4^+$ lymphocytes

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	2100 ± 266	2152 ± 296	NS
Mesenteric lymph node	12206 ± 1123	14407 ± 2654	NS
Ileal Peyer's patch	3486 ± 441	7006 ± 1304	P=.009

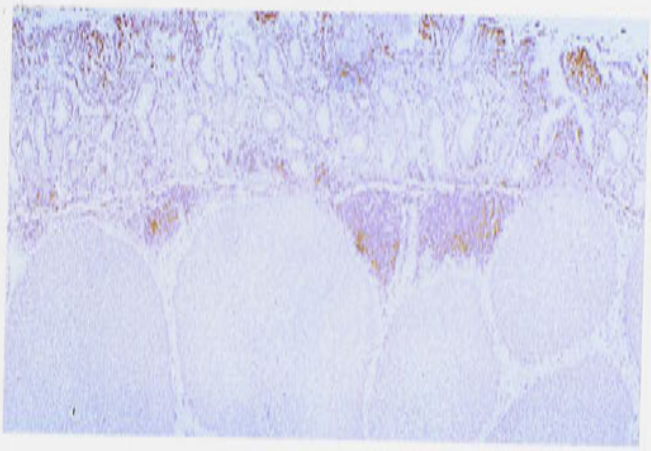
Table- 3.2: $CD4^+$ lymphocytes expressed as a percentage of total leukocyte population

Tissue	Mean ± S.E. (percentage)		Sig.
	In utero antigen-exposed	Control	
Jejunum	11.78 ± 2.2	8.76 ± 1.1	NS
Mesenteric lymph node	29 ± 4	30.1 ± 4.6	NS
Ileal Peyer's patch	3.32 ± 0.47	6.84 ± 1.4	P=.015

Fig-A: $CD4^+$ lymphocytes within the IPP of *in utero* antigen-exposed lambs. The area staining for $CD4^+$ lymphocytes was significantly smaller within the IPP of *in utero* antigen-exposed lambs in comparison with control lambs as assessed by image analysis.

Fig-B: $CD4^+$ lymphocytes within the IPP of control lambs. The area staining for $CD4^+$ lymphocytes was significantly greater within the IPP of control lambs in comparison with *in utero* antigen-exposed lambs as assessed by image analysis.

Fig-A:



80 μm

Fig-B:

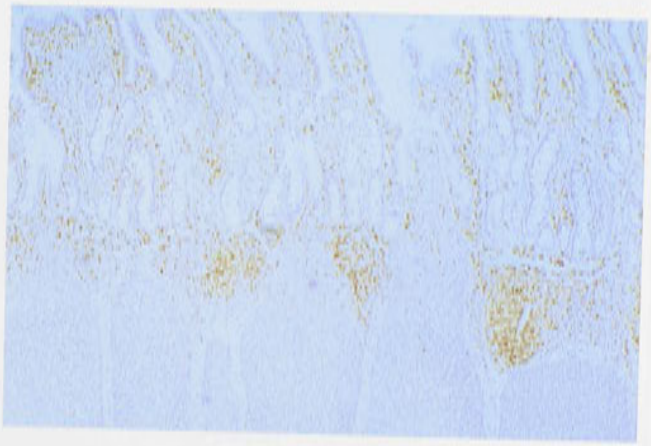


Fig-3.3: Frequency of occurrence of $CD4^+$ lymphocytes in jejunum

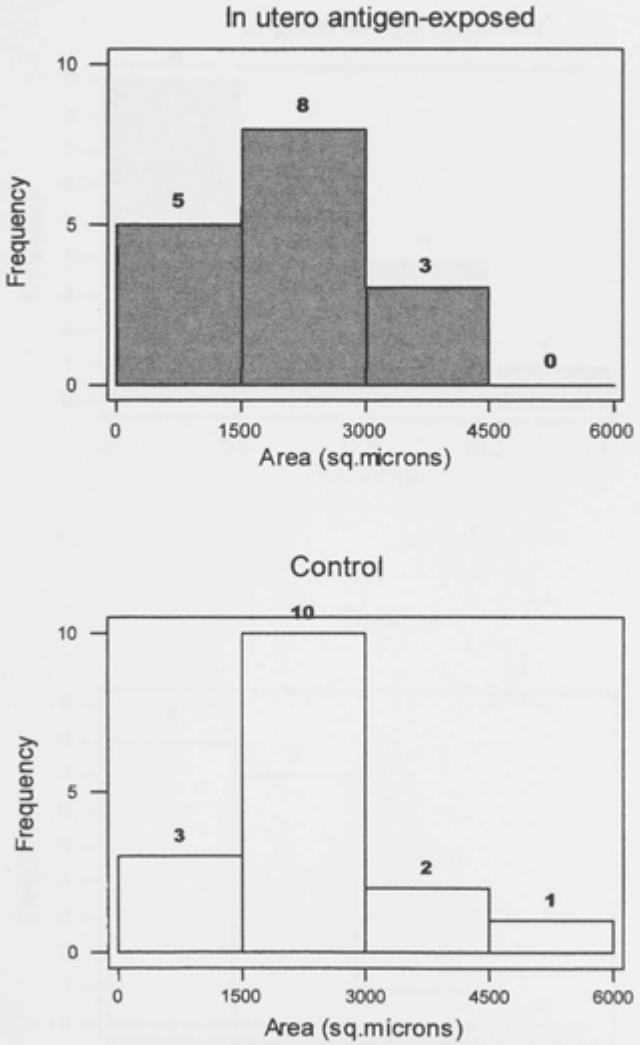
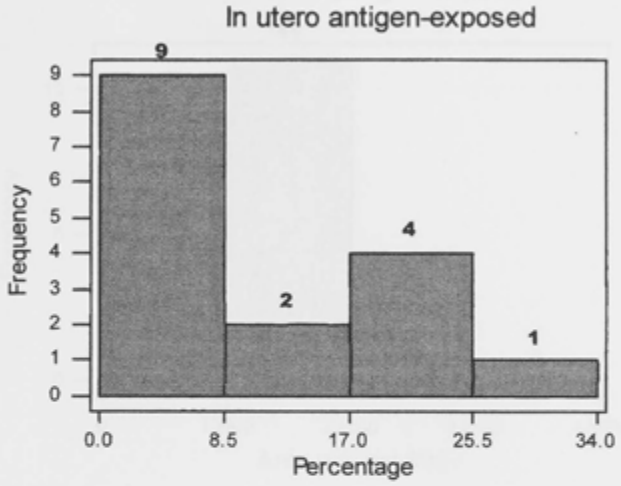


Fig-3.4: Frequency of occurrence of $CD4^+$ lymphocytes in the jejunum expressed as a percentage of total leukocyte population



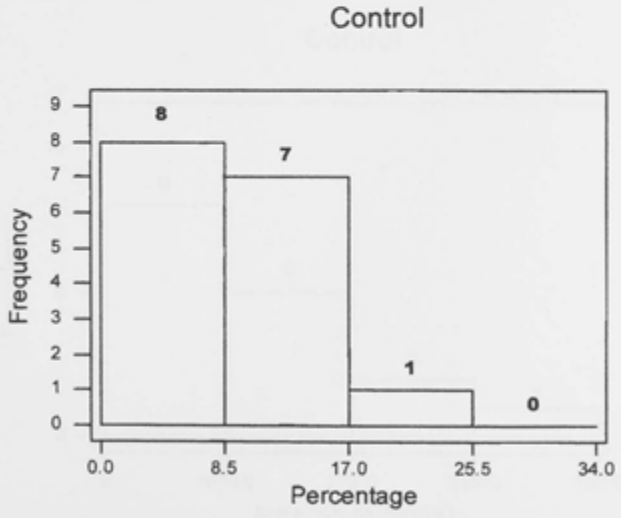
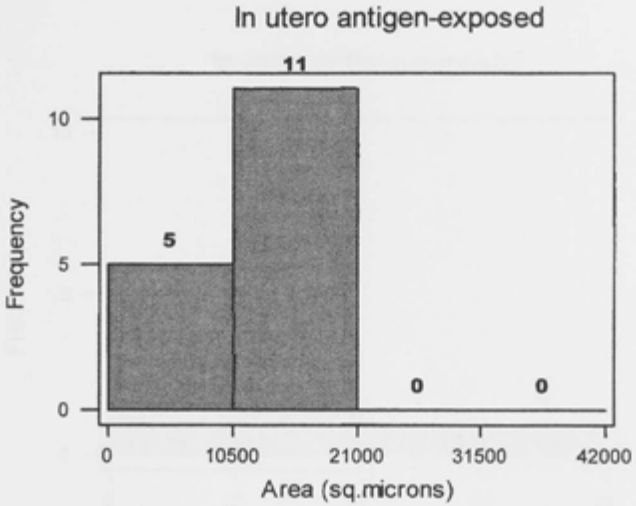


Fig-3.5: Frequency of occurrence of $CD4^+$ lymphocytes in the MLN



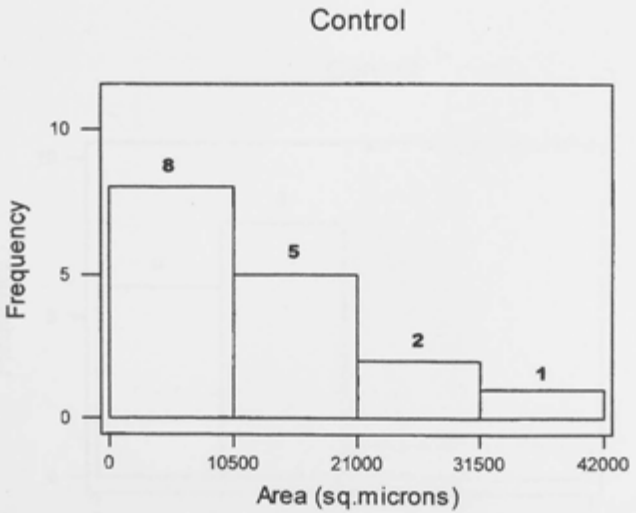
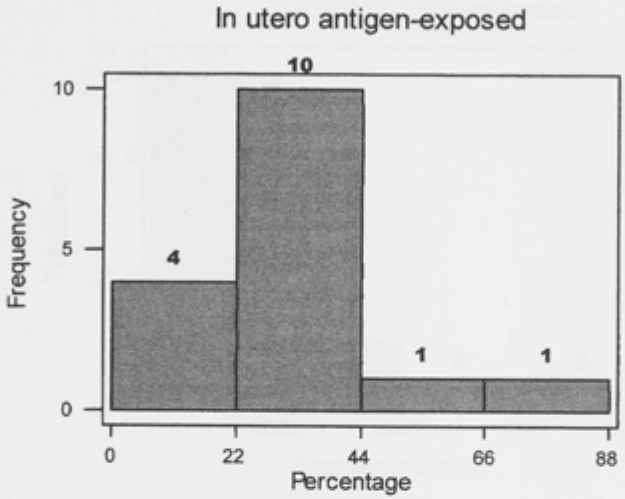


Fig-3.6: Frequency of occurrence of $CD4^+$ lymphocytes in the MLN expressed as a percentage of total leukocyte population



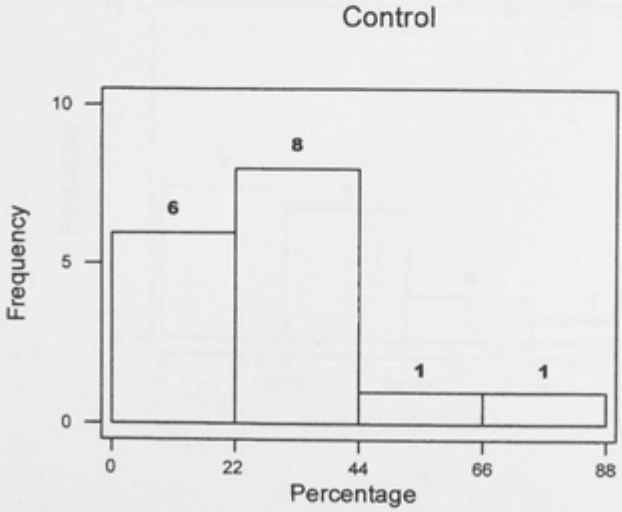
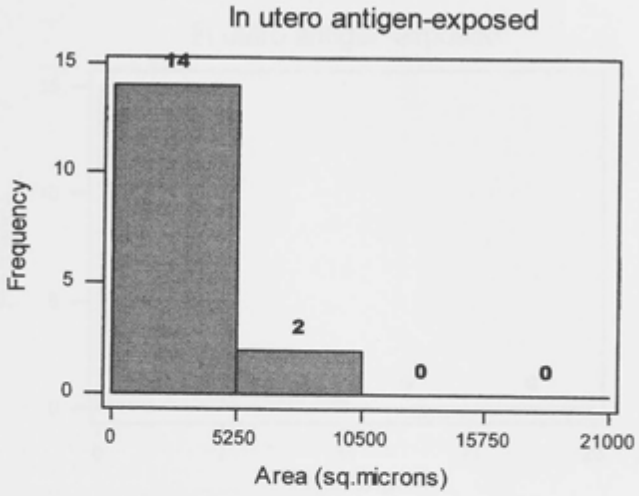


Fig-3.7: Frequency of occurrence of $CD4^+$ lymphocytes in the IPP



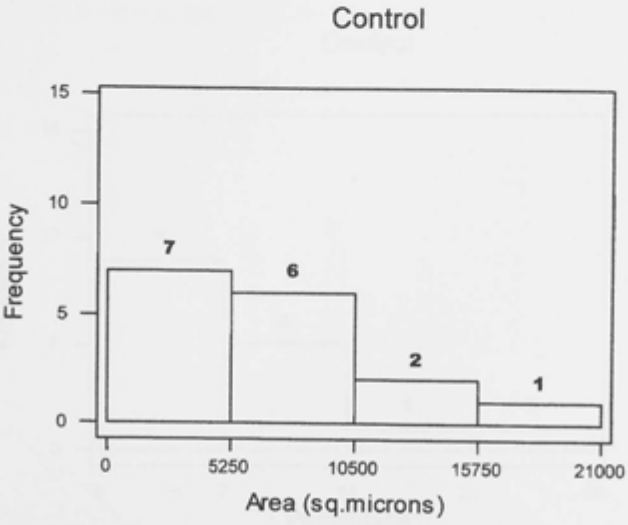
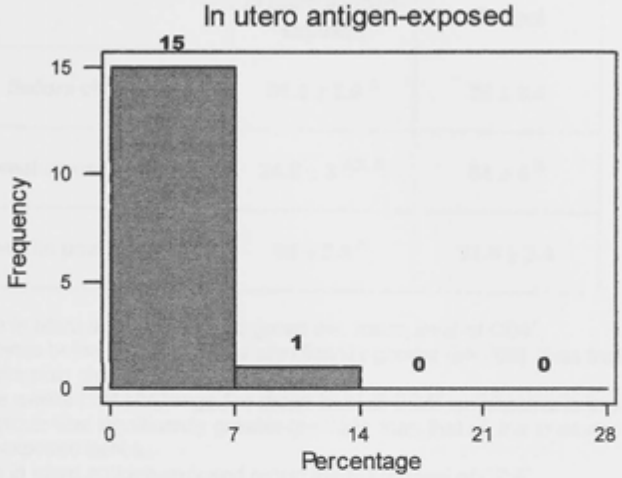


Fig-3.8: Frequency of occurrence of $CD4^+$ lymphocytes in the IPP expressed as a percentage of total leukocyte population



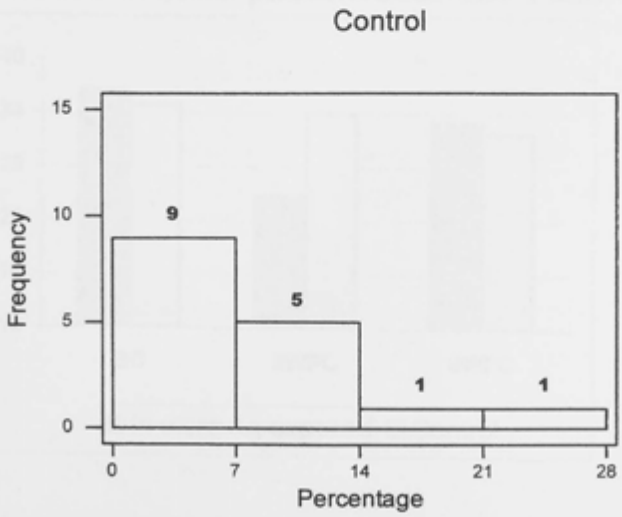
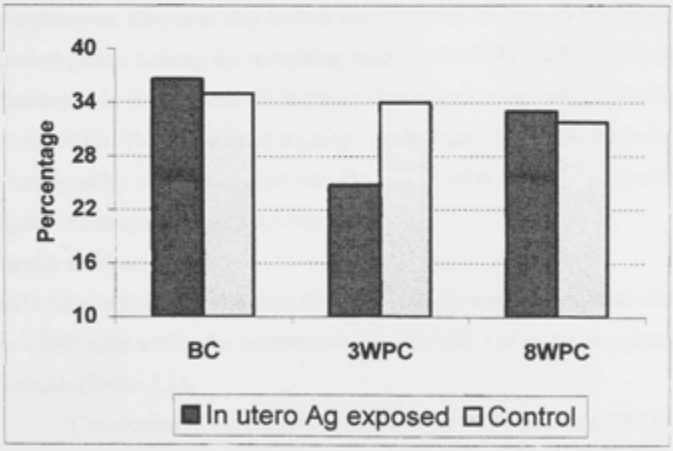


Table-3.9: FACS analysis of peripheral blood- $CD4^+$ T cells

Time	Mean ± S.E. (percentage)	
	In utero antigen-exposed	Control
Before challenge	36.6 ± 2.9 [A]	35 ± 3.4
3 weeks post-challenge	24.8 ± 3 [A,B,C]	34 ± 4 [B]
8 weeks post challenge	33 ± 2.3 [C]	31.8 ± 2.4

A: In the *in utero* antigen-exposed group the mean level of $CD4^+$ lymphocytes before challenge was significantly greater (p=.005) than that at 3 weeks post challenge.
B: Three weeks post challenge the mean level of $CD4^+$ lymphocyte in the control group was significantly greater (p=.039) than that of the *in utero* antigen-exposed lambs.
C: In the *in utero* antigen-exposed group the mean level of $CD4^+$ lymphocytes at 8 weeks post challenge was significantly greater (p=.021) than that at 3 weeks post challenge.

Fig-3.10: FACS analysis of peripheral blood- $CD4^+$ T cells



lymphocytes within the duodenal lamina propria of naïve or immune animals. On the contrary, Gorrell *et al.* (1988b) observed that two out of four naïve sheep superinfected with 50,000 larvae exhibited marked reduction in the number of $CD4^+$ lymphocytes within the duodenal mucosa.

$CD4^+$ T cells in newborn human infants are immature compared to adult blood derived $CD4^+$ T cells. This is likely to have a major effect on the type of immune response generated to foreign antigens. They have greater responsiveness to cytokines such as IL-1 β and IL-4 while adult cells exhibit diminished responses to them (Reen, 1998). Reen (1998) has suggested that the human neonatal $CD4^+$ T cell is in a maturation stage befitting a 'recent thymic emigrant'. The stage of maturation of $CD4^+$ lymphocytes of perinatal lambs remains a matter of speculation with no apparent published evidence. Thus, any attempts to address this issue would be worthwhile.

RESULTS

Jejunum:

Histology: Positive lymphocytes were usually observed to be infiltrating into the villi. Very often, the cells tended to be clustered in small patches (2-4 cells) towards the apical end of the mucosal villi. Almost all the animals (15/16) from the *in utero* antigen-exposed group had this distribution pattern of $CD4^+$ lymphocytes. However, this feature was observed in only 8/16 animals from the control group. Among the remaining animals, the $CD4^+$ cells were randomly distributed in the villi and the lamina propria, very often being close to the base of the villus. The intensity of staining usually ranged from moderate to high. Occasionally, cells infiltrating into the villi of some animals were extremely lightly staining with hazy borders.

Image analysis:

a)Total positive area staining for $CD4^+$ lymphocytes: The mean area occupied by $CD4^+$ cells within the jejunum was almost identical in the two groups of animals (Table-3.1).

Correlation of the frequency distribution of the animals with the area occupied by $CD4^+$ cells did not reveal any appreciable differences between the two groups (Fig-3.3).

b)$CD4^+$ lymphocytes expressed as a percentage of the total leukocyte population: The area stained by $CD4^+$ cells was divided by the total area stained with the pan leukocyte marker CD45 and then expressed as percentage. There was a trend for the mean percentage of $CD4^+$ lymphocytes in the jejunum to be marginally higher in the *in utero* antigen-exposed lambs in comparison with control animals (Table-3.2). This difference was not significant statistically.

The frequency distribution of $CD4^+$ lymphocytes expressed as a percentage of the total leukocyte population, was distinctly different in the two groups (Fig-3.4). Among the *in utero* antigen-exposed animals there were only two animals (Fig-3.4) in which $CD4^+$ lymphocyte levels fell within the range of >8.5 and <17 %. Among the control group of animals (Fig-3.4) 7/16 animals fell within the same range (>8.5 and <17 %). The number of animals having CD4 levels >17% was considerably higher (5/16) in the *in utero* antigen-exposed animals in comparison to control animals (1/16).

Mesenteric lymph node:

Histology: Few histological differences were immediately apparent between the two groups of animals. The positive cells were clustered in patches between the trabeculae of the medullary region. The positive cells were usually stained with moderate intensity. However, on rare occasions, positive cells tended to stain very lightly and had hazy boundaries.

Image analysis:

a) Total positive area staining for $CD4^+$ lymphocytes: The mean area that stained for $CD4^+$ cells was not significantly different between the two groups. There was a marginal tendency for the stained area to be higher in the control group of animals (Table-3.1).

The frequency distribution of animals in relation to the area occupied by $CD4^+$ cells in the MLN revealed a few differences between the two groups (Fig-3.5). A majority of the animals (11/16) in the *in utero* antigen-exposed group (Fig-3.5) had a $CD4^+$ area that fell within the range of >10,500 and <21,000 sq.μ. In the control group there were fewer (5/16) animals within this range (>10,500 and <21,000 sq.μ). None of the animals from the *in utero* antigen-exposed group had an area >21,000 sq.μ. In contrast, among control animals there were three animals with an area >21,000 sq.μ (Fig-3.5).

b)$CD4^+$ lymphocytes expressed as a percentage of the total leukocyte population: The mean level of $CD4^+$ lymphocytes, expressed as a percentage of the total leukocyte population, was almost identical in the two groups of animals (Table-3.2).

The frequency distribution of $CD4^+$ lymphocytes, expressed as a percentage of the total leukocyte population, was not appreciably different between the two groups (Fig-3.6).

Ileal Peyer's patch:

Histology: There were appreciable histological differences between the two groups of lambs. Among *in utero* antigen-exposed lambs, the average size of the interfollicular region staining for $CD4^+$ was considerably smaller than in the controls. The positive cells in the interfollicular zone tended to be distributed in a loose mesh like structure. There were many cells scattered in the boundary of the interfollicular zone and infiltrating into the mucosal villi. Among the control animals, the interfollicular region staining for $CD4^+$ cells was considerably more extensive. The positive cells in the interfollicular zone were much more tightly packed than those observed in animals exposed to the antigen *in utero*. Fewer (5/16) control animals had $CD4^+$ lymphocytes infiltrating the mucosa in comparison with *in utero* antigen-exposed lambs (10/16). Positive lymphocytes were considerably darker staining in comparison with those observed in the MLN and, to a lesser extent in the jejunum. Occasionally $CD4^+$ lymphocytes were seen infiltrating into the connective tissue capsule of the follicles. A few $CD4^+$ lymphocytes were also seen *inside* the follicles in close association with B lymphocytes. Among the *in utero* antigen-exposed group 8/16 lambs compared with 7/16 from the control group, had a few $CD4^+$ lymphocytes infiltrating into the follicles of the IPP.

Image analysis:

a) Total positive area staining for $CD4^+$ lymphocytes: The area staining for $CD4^+$ lymphocytes in the IPP was significantly greater ($p=.009$) in the control group of animals in comparison with the *in utero* antigen-exposed animals (Table-3.1).

The frequency distribution of the area staining for $CD4^+$ lymphocytes was also distinctly different between the two groups of lambs (Fig-3.7). Among the *in*

utero antigen-exposed lambs (Fig-3.7), the majority of animals (14/16) had a positive area <5250 sq.μ. In contrast, in the control group there were only 7/16 animals (Fig-3.7) with the same area of $CD4^+$ cells (<5250 sq.μ.). There were only two animals with a positive area >5250 sq.μ in the *in utero* antigen-exposed group. On the contrary, the majority of animals (9/16) from the control group (Fig-3.7) had a positive area >5250 sq.μ.

b)$CD4^+$ lymphocytes expressed as a percentage of the total leukocyte population: The mean level of $CD4^+$ lymphocytes expressed as a percentage of the total leukocyte population was significantly greater (p=.015) in the control group of animals in comparison with the *in utero* antigen-exposed animals (Table-3.2).

The frequency distribution of $CD4^+$ lymphocytes expressed as a percentage of the total leukocyte population differed between the two groups (Fig-3.8). In the *in utero* antigen-exposed lambs, the majority of animals (15/16) had <7 % of the total lymphocytes in the IPP positive for CD4. In comparison, only 9/16 animals from the control group (Fig-3.8) had the same percentage levels (<7 %) of $CD4^+$ cells. There were a much greater number of animals (7/16) in the control group with >7% of $CD4^+$ lymphocytes in the IPP. Among the *in utero* antigen-exposed lambs there was only one animal with the same level (>7%) of $CD4^+$ lymphocyte.

FACS analysis of $CD4^+$ peripheral blood lymphocytes: Significant changes in the $CD4^+$ lymphocyte levels of the peripheral blood in response to challenge with *T. colubriformis* were only evident in the *in utero* antigen-exposed group of animals (Table-3.9 and Fig-3.10). In the control group, the percentage levels of $CD4^+$ lymphocytes did not exhibit significant change (Table-3.9 and Fig-3.10). Three weeks post-challenge there was a marked drop in the number of $CD4^+$ lymphocytes in the peripheral circulation of *in utero* antigen-exposed animals. Consequently, in the *in utero* antigen-exposed group the mean level of $CD4^+$ lymphocytes before challenge was significantly greater (p=.005) than that at three weeks post challenge. Likewise, at three weeks post challenge the mean level of $CD4^+$ lymphocytes in the control group was significantly greater (p=.039) than that of the *in utero* antigen-exposed lambs. At eight weeks post-challenge, $CD4^+$ levels were no longer depressed in the *in utero* antigen-exposed

lambs. Thus, the mean level of $CD4^+$ lymphocytes at eight weeks post challenge was significantly greater ($p=.012$) than that observed three weeks post-challenge.

DISCUSSION

Protective immunity against gastrointestinal nematode parasites is $CD4^+$ T cell dependent (Finkelman *et al.*, 1997). The present study has revealed that the $CD4^+$ lymphocyte response in the gut and the peripheral circulation can be significantly altered by prior *in utero* oral exposure to antigen. Three weeks after challenge with live larvae, there was a significant drop in the $CD4^+$ lymphocyte levels in the peripheral circulation of *in utero* antigen-exposed lambs. However, this drop in the $CD4^+$ levels was not observed in the control lambs which had not been previously exposed to parasitic antigen *in utero*. It appears that three weeks after the onset of infection, among *in utero* antigen-exposed lambs, the peripheral $CD4^+$ lymphocytes were being rapidly depleted from the periphery and retained in the target site of infection namely the gut. The fact that this drop was evident only in the *in utero* antigen-exposed group suggests that a considerable number of these cells might have been memory $CD4^+$ lymphocytes. It appears that the gut mucosal immune system of the foetal lamb at 100 days, has reached a developmental stage at which $CD4^+$ lymphocytes can be altered by oral exposure to a foreign antigen. These antigen-modulated $CD4^+$ lymphocytes seem to retain the memory of their previous antigenic exposure as indicated by a rapid clearance of these cells from the peripheral circulation following challenge.

The anamnestic response of the peripheral $CD4^+$ lymphocytes to the *Trichostrongylus* infection was not accompanied by an efficient effector response ie. a lowered worm burden in the jejunum of *in utero* antigen-exposed lambs. This discrepancy could be partly explained by the findings of Kambara and McFarlane (1996). They observed that, among older animals which are generally resistant to *Trichostrongylus*, $CD4^+$ levels in the peripheral circulation increased after exposure to the parasite. A decrease in the number of peripheral $CD4^+$ lymphocyte was evident after infection with the *Trichostrongylus* only among younger lambs. In the present study, when the *in utero* antigen-exposed lambs had a significant drop of $CD4^+$ lymphocytes levels in the peripheral circulation three weeks after challenge, the lambs had a marginally higher worm count in the gut at sacrifice.

The IPP was the anatomical site in the gastrointestinal tract where $CD4^+$ levels were significantly different between the two groups of lambs. Among *in utero* antigen-exposed lambs the average size of the interfollicular region was considerably smaller with $CD4^+$ lymphocytes distributed in a loose mesh-like structure. In direct contrast, in the control lambs the interfollicular region was considerably larger with $CD4^+$ cells tightly packed within the zone. The $CD4^+$ lymphocyte levels, expressed either as total positive area or as a percentage of total leukocyte population, were significantly higher in the IPP of control lambs in comparison with these values in the *in utero* antigen-exposed lambs. Thus the IPP of *in utero* antigen-exposed lambs appear to have been depleted of a significant number of $CD4^+$. It is possible that the prior *in utero* antigen-exposure might have altered the response of $CD4^+$ lymphocytes within the IPP to the parasitic antigen. On re-exposure to the homologous antigen in postnatal life, these cells appear to have rapidly migrated away from the IPP in an anamnestic fashion, perhaps to the location of the parasite.

Within a *Haemonchus contortus* resistant strain of sheep, the resistance can be completely abrogated by the depletion of $CD4^+$ lymphocytes (Gill *et al.*, 1993). There is also a strong suggestion that $CD4^+$ lymphocytes might play an important role in the immunity to *Trichostrongylus* among sheep (McClure *et al.*, 1992). The control group of lambs were able to achieve a marginally lower worm burden in the jejunum although a considerable number of $CD4^+$ lymphocytes were still retained in the IPP. In direct contrast, *in utero* antigen-exposed lambs had a marginally higher worm burden in the gut accompanied by a rapid depletion of $CD4^+$ lymphocytes from the IPP. Thus, it appears that marked depletion of $CD4^+$ lymphocytes from the IPP was not very helpful to the *in utero* antigen-exposed lambs in achieving a lower worm count in the gut.

It is possible that the $CD4^+$ lymphocytes depleted from the IPP of *in utero* antigen-exposed lambs might have migrated into tissues such as the jejunum and MLN. The total $CD4^+$ areas within the jejunum and MLN were almost identical in the two groups of lambs and thus it appears that $CD4^+$ lymphocytes had not migrated from the IPP into these tissues. However, when $CD4^+$ levels in the jejunum were expressed as a percentage of the total leukocyte population, a marginally higher level was evident in the *in utero* antigen-exposed

group of lambs. Thus it is possible that at least some of the $CD4^+$ cells depleted from the IPP of the *in utero* antigen-exposed group might have migrated into the target site of infection, the jejunum. Increased numbers of $CD4^+$ lymphocytes have been reported in the jejunum of immune sheep during the second phase of rejection of *Trichostrongylus* from the gut, which occurs between five and ten days post exposure (McClure *et al.*, 1992). The findings of Gorrell *et al.* (1988b) differ from the findings of the present work as well as from those of McClure *et al.* (1992). They observed that challenge with 20,000 or fewer *Trichostrongylus* larvae did not alter the number of $CD4^+$ lymphocytes within the lamina propria of the jejunum. The sensitisation protocol as well as the challenge dose employed by Gorrell *et al.* (1988b) was distinctly different from the present study and hence direct comparison might not be appropriate.

Apart from the marginally increased percentage levels of $CD4^+$ lymphocytes in the jejunum, the *in utero* antigen-exposed group had another important histological difference from the control group of animals. In the majority of *in utero* antigen-exposed animals, the $CD4^+$ lymphocytes were clustered towards the apical end of the villi as if actively responding to the infection. However, this clustering of $CD4^+$ lymphocytes into the apical end of the villi was only observed in 50 % of animals from the control group. Even with the marginally lower $CD4^+$ levels in the jejunum and their apparently weaker histological response to the parasite, the control animals were able to achieve a lower worm count in the gut in comparison with *in utero* antigen-exposed lambs. Thus, in the parasite infected gut the determining factors might not be the actual number of $CD4^+$ lymphocytes but their functional capacity and the effectiveness with which they interact with other components of the immune system. Furthermore, the histological differences in the CD4 response in the jejunum of the two groups raises the possibility that a more vigorous response might not be beneficial to the host.

There is also a possibility that the $CD4^+$ lymphocytes from the IPP of *in utero* antigen-exposed animals might have migrated into other regions of the body, such as the peripheral lymphocyte compartment. The published evidence of Premier *et al.* (1996) does not support the current belief that lymphocytes activated at a particular site preferentially migrate back to the same or a similar

site (mucosal or peripheral) (Hall *et al.*, 1977; Mackay, 1993). Premier *et al.* (1996) inoculated keyhole limpet haemocyanin into the rectal mucosa of sheep and observed that the antigen-specific $CD4^+$ lymphocytes which were induced, preferentially migrated to the peripheral lymph nodes such as the popliteal and prescapular.

One of the important criteria for identifying the IPP as a primary lymphoid organ has been the low level of $CD4^+$ T lymphocytes reported in the tissue. Studies by Hein *et al.* (1989) and Aleksandersen *et al.* (1990) have consistently reported that $CD4^+$ T lymphocytes within the IPP represent less than 1% of the total lymphocyte population. However, the present study did not find any evidence to support their observations. Even in the *in utero* antigen-exposed lambs which had very low levels of $CD4^+$ lymphocytes in the IPP the mean level was 3.32 ± 0.47 %. In control lambs the percentage of $CD4^+$ T lymphocytes in the IPP was more than twice (6.84 ± 1.4 %) that observed among *in utero* antigen-exposed lambs. This difference may be a reflection of the use of different approaches to quantify lymphocytes. Both Hein *et al.* (1989) and Aleksandersen *et al.* (1990) carried out flow cytometry analysis of the dissociated IPP cells. Furthermore, their inferences were based on investigations with a very limited number of uninfected animals. Alternatively, there may be a breed difference in $CD4^+$ T cell content of IPP.

Between 40-50 % of animals from both the groups had $CD4^+$ lymphocytes infiltrating into the follicles of the IPP. Although their numbers were few it is possible that $CD4^+$ T cells might be involved in primary B cell development in the IPP of sheep or that the IPP might have the capacity to function as a secondary as well as a primary lymphoid organ.

CD8

INTRODUCTION

The cluster of differentiation antigen CD8 is co-expressed with CD4 on the majority of immature lymphocytes in the thymus (Reinherz & Schlossman, 1980). As thymic T cells acquire immunocompetence they segregate into $CD4^+$ and $CD8^+$ subpopulations (Reinherz *et al.*, 1980). In mature T cells, CD8 defines a subpopulation of lymphocytes the receptors of which are usually restricted by class I MHC (Swain, 1983). The functional homologue of the CD8 molecule in sheep is OvCD8 (Keech & Brandon, 1991b). In mouse and man the CD8 antigen is a doublet which can exist as either a homodimeric or heterodimeric form. The heterodimeric form is found on cells of the thymus in man while the homodimeric form occurs on cells in the peripheral circulation. In contrast, in sheep the higher molecular form of the antigen is found on peripheral lymphocytes (Maddox *et al.*, 1985b).

Cytotoxic role of $CD8^+$ T cells: The major functional role attributed to $CD8^+$ cells relates to cytotoxicity. This is effected by a Ca^{2+} / Perforin dependent mechanism and, to a lesser extent, via Fas. Cytotoxic cells are considered to be important in recovery from intracellular infections and play a vital role in virus clearance in a variety of species (Andrew *et al.*, 1995; Martins *et al.*, 1988; Yap *et al.*, 1978). They often mediate cross-protective immunity (Braciale, 1979). However, it is not known whether cytotoxic cells have a protective role in infections with larger multicellular organisms such as nematode parasites. In situations of massive microbial load in the gastrointestinal tract, unrestricted cytotoxicity on the part of these cells might be detrimental to the survival of the host. Consequently, an understanding of the role of $CD8^+$ cells in gastrointestinal tract parasitic infections is of particular interest.

Regulatory role of $CD8^+$ T Cells: $CD8^+$ lymphocytes are thought to play an important role in the regulation of the immune response. "Classical" $CD8^+$ lymphocytes secrete a Th1-like cytokine profile which includes interferon γ and IL-2 . There is a strong bias for $CD8^+$ cells to differentiate into interferon γ producers (Mosmann & Sad, 1996). The subset of $CD8^+$ cells which differentiate in this way is called Tc1. Another subset of $CD8^+$ lymphocytes, the Tc2 or Th2 subset, the differentiation of which can be induced especially by IL-4, is

considered to be important in immune responses. Tc2 cells can secrete the IL-4, IL-5 and IL-10 classes of interleukins. After commitment of $CD8^+$ T cells to either Tc1 or to Tc2 phenotypes, neither subset can re-convert to the other cytokine-secreting subset (Mosmann & Sad, 1996).

As responses generated by Tc2 cells are generally immunosuppressive, these cells are also referred to as the $CD8^+$ suppressor cells. It has been shown that $CD8^+$ T cells from lepromatous leprosy lesions can be activated to suppress $CD4^+$ T cell proliferation *in vitro* (Modlin *et al.*, 1986). Likewise, in the bovine species, suppressor $CD8^+$ T cells have been reported to alter the immune responsiveness during the postpartum period (Shafer-Weaver & Sordillo, 1997).

$CD8^+$ T lymphocytes in the gastrointestinal tract: The distribution of $CD8^+$ T cells in the gastrointestinal tract depends upon numerous factors such as age, species, the region of the gut examined and presence or absence of infection in the gut. $CD8^+$ T cells occur in the epithelium, lamina propria and the draining lymph nodes.

Many T lymphocytes occur in the epithelium of the gastrointestinal tract. These intraepithelial lymphocytes (IEL) are thought to play an important role in the first line of defence against an extensive range of external antigens including harmful pathogenic microbes that pass through the intestine. At least some of these T cells develop in the intestinal mucosa independently of the thymus (Nanno *et al.*, 1998). They have cytotoxic potential and many contain cytoplasmic granules. The capacity of IEL to process antigens from the lumen of the intestine is likely to be limited by the reduced presence of processing enzymes like cathepsin B and I. Consequently, production of peptides too large to fit into the antigen binding groove of MHC II molecule results. Such larger antigenic peptides are more likely to bind to CD1d molecules and preferentially stimulate $CD8^+$ rather than $CD4^+$ T cells as occurs in the systemic immune response (Mayer, 1997).

The phenotype of IEL varies with species. In mice and avian species the major subpopulation of intraepithelial lymphocytes are $\gamma\delta$ T cells. In calves they are predominantly $\gamma\delta$ or $CD8^+$ T cells (Waters *et al.*, 1995). As in other species, $CD8^+$ cells predominate among the intraepithelial lymphocytes of sheep (Gyorffy *et al.*, 1992). Some of these $CD8^+$ IEL have the $\alpha\beta$ T cell receptor while others

have the γδ T cell receptor. In lambs, IEL are detectable in the duodenum of foetal lambs (Gorrell *et al.*, 1988b). The number of IEL does not increase during the first week after birth, but, by 9 weeks of age there is a 3-4 fold increase in the duodenal epithelium (Gorrell *et al.*, 1988b).

The lamina propria of the gastrointestinal tract contains a large population of $CD8^+$ T cells . The proportion of T cells in the lamina propria that are $CD8^+$ is similar to that in the peripheral blood. Approximately 50% of the lamina propria $CD8^+$ T cells are $CD28^+$ and possess cytolytic capacity although evidence for antigen-specific cytolytic function in the human intestine is lacking. The other major $CD8^+$ T cell subgroup carries the $CD11^+$ suppressor phenotype (McGowan *et al.*, 1997).

A rapid increase in the number of $CD8^+$ T cells in the duodenal villous lamina propria occurs in lambs immediately after birth. Eight days after birth, lambs have a $CD8^+$ T cell population that is approximately 70% of that in adult sheep. However, full adult numbers of these cells are not attained until 5 months of age (Gorrell *et al.*, 1988b). $CD4^+$ T lymphocytes are invariably present in close association with $CD8^+$ T cells in the lamina propria. The ratio of the two cell types to each other varies with the location in the gut, age and disease status (Josefsen & Landsverk, 1996; McGowan *et al.*, 1997).

In the Peyer's patches $CD8^+$ T cells are confined to the interfollicular region (Hein *et al.*, 1989; Press *et al.*, 1991). The $CD8^+$ T cells within the lamina propria of the gut are thought to be replenished by precursors which originate from the Peyer's patches (Bloom & Boedeker, 1996). During a secondary immune response the population of $CD8^+$ T cells can be repopulated from the Peyer's patches or alternatively can be locally expanded.

Hein *et al.* (1989) analysed the $CD8^+$ lymphocyte population in the ileal Peyer's patches (IPP) and the jejunal Peyer's Patches (JPP) of five lambs aged between 5 days to 16 weeks using flow cytometry and immunoperoxidase staining of the frozen tissue to quantify the levels of $CD8^+$ T cells. Cell suspensions prepared from IPP contained less than 1 % $CD8^+$ T cells and those from the JPP contained approximately 2 %. Examination of sections revealed that $CD8^+$ T cells occurred exclusively in the interfollicular region and were not found

within the JPP follicles. The distribution of $CD8^+$ T cells within the IPP was similar (Hein *et al.*, 1989).

Aleksandersen *et al.* (1990) also examined the cellular composition of lymphoid follicles within the gastrointestinal tract of sheep. They found that the large intestine Peyer's patches were generally similar to the JPP, the $CD8^+$ T cells levels in both locations being almost identical (3.8-7.95 %) . Both tissues have numerous $CD8^+$ and $CD4^+$ T cells in the interfollicular region. In contrast, in the IPP the number of $CD8^+$ T cells was less than 1 % of the total lymphocyte population. These observations were based on FACS analysis of dissociated cells suspensions of 3 and 2 lambs respectively from the large intestine Peyer's patches and IPP/JPP (Aleksandersen *et al.*, 1990). Consequently, one should remain cautious in drawing conclusions from this study and that of Hein *et al.* (1989). Within the paracortical region of the mesenteric lymph node of sheep, large numbers of $CD8^+$ T cells occur (Keech & Brandon, 1991b).

$CD8^+$ T lymphocyte response to *Trichostrongylus* infection in sheep : The number of reports on this subject is very limited. Gorrell *et al.* (1988b) observed that sheep (30-34 weeks postnatally) exhibited dose-dependent changes in the $CD8^+$ T cell population. The administration of 20,000 or fewer larvae of *T. colubriformis* did not alter the number of $CD8^+$ T cells within the duodenum or jejunum irrespective of whether the sheep were immune to the parasite or not. However, naive sheep challenged with a massive dose of 50,000 larvae exhibited a marked reduction in the number of $CD8^+$ T cells in the lamina propria of the duodenal tissue taken within one metre from the pylorus. In contrast, jejunal tissue collected five metres distal to the pylorus did not manifest any change, four weeks after challenge. Gorrell *et al.* (1988b) did not observe any changes in the $CD8^+$ T cell population of the mesenteric lymph node in these sheep.

McClure *et al.* (1992) have detected differences between the $CD8^+$ T lymphocyte responses in the jejunum of naive and immune sheep following infection with *Trichostrongylus colubriformis*. Among susceptible sheep, there was only a slight increase in the number of $CD8^+$ cells infiltrating the jejunum three days after infection with the parasite. This mildly elevated level persisted for 14 days without any further increase. In contrast, among immune sheep, larval challenge was followed by a much greater increase in the number of $CD8^+$ T

cells within the lamina propria and mucosa of the jejunum. This rise was observed from the third day after challenge and persisted until the fifth day. After this, the frequency of $CD8^+$ T cells in the jejunum declined to pre-challenge levels during the period from the 5^{th} to the 14^{th} day post-challenge (McClure *et al.*, 1992).

$CD8^+$ T cell levels in sheep infected with *Trichostrongylus* are also influenced by the nutritional status and the age of the animal (Kambara & McFarlane, 1996). Irrespective of their nutritional status, older animals (31-51 weeks) showed a significant increase in the number of $CD8^+$ cells in the peripheral lymphocyte population following repeated immunisation with *Trichostrongylus* larvae whereas, in younger animals (8-26 weeks), there was a marginal decrease. Pernthaner *et al.* (1996) have also reported a significant increase in the proportion of $CD8^+$ T cell levels in the peripheral blood during the first four weeks following infection with *T. axei*. They challenged *Trichostrongylus* resistant and susceptible lambs with 10,000 larvae but found no difference between the two genetic lines of sheep in terms of $CD8^+$ T cell dynamics in the peripheral circulation. Similar age dependent differences in $CD8^+$ T levels were observed in the mesenteric lymph node (Pernthaner *et al.*, 1996).

McClure *et al.* (1996) have specifically addressed the role played by $CD8^+$ cells in the development of protective immunity in sheep against infection with *Trichostrongylus* . They administered monoclonal antibodies against $CD8^+$ lymphocytes and γ interferon during the induction phase of the immune response with the aim of depleting the sheep of $CD8^+$ cells or interferon γ. This strategy was successful to a limited extent as, whilst $CD8^+$ cells could be depleted from the peripheral circulation, intestinal levels nevertheless remained unaffected. It was concluded that prolonged administration of monoclonal antibodies against $CD8^+$ cells and γ interferon significantly increased protection during primary infection and following challenge. That is, suppression of $CD8^+$ cells appeared to favour recovery from *Trichostrongylus* infection (McClure *et al.*, 1996).

Table-4.1: Total positive area - $CD8^+$ T lymphocytes

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	758 ± 140	482.5 ± 73	P=.048
Mesenteric lymph node	17258 ± 2052	19043 ± 3296	NS
Ileal Peyer's patch	4050 ± 566	6657 ± 1415	P=.05

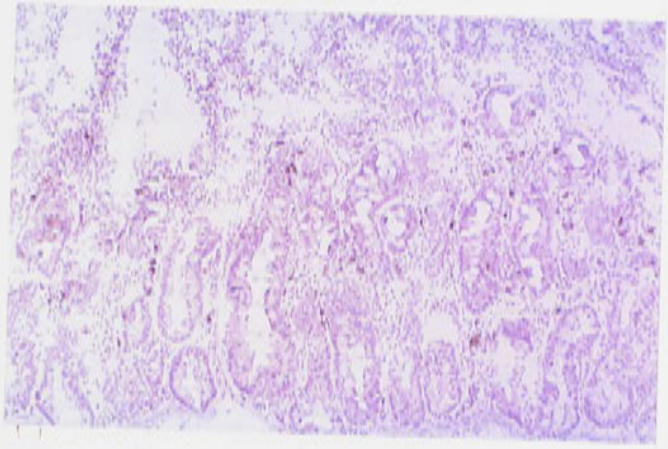
Table-4.2: Percentage of $CD8^+$ lymphocytes to the total leukocyte population

Tissue	Mean ± S.E. (percentage)		Sig.
	In utero antigen-exposed	Control	
Jejunum	3.87 ± 1	1.83 ± 0.24	P=.032
Mesenteric lymph node	41.6 ± 6.5	38.8 ± 5.1	NS
Ileal Peyer's patch	3.8 ± 0.6	6.6 ± 1.5	P=.05

Fig-C: $CD8^+$ cells distributed within the jejunal mucosa of *in utero* antigen-exposed lambs. The area staining for CD8 was significantly greater in the *in utero* antigen-exposed group in comparison with control lambs as assessed by image analysis.

Fig-D: $CD8^+$ cells distributed within the jejunal mucosa of control lambs. The area staining for CD8 was significantly lesser in the control group in comparison with *in utero* antigen-exposed lambs as assessed by image analysis.

Fig-C:



40 μm

Fig-D:

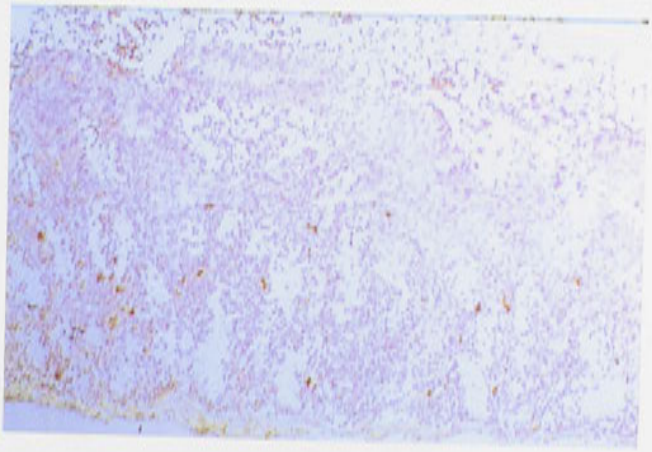
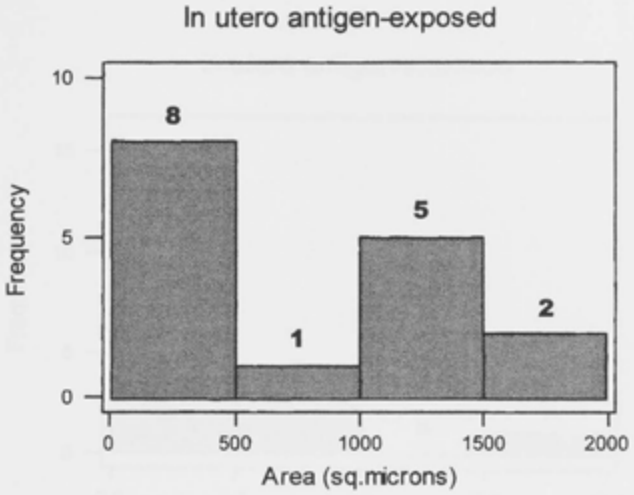


Fig-4.3: Frequency of occurrence of $CD8^+$ cells in the Jejunum



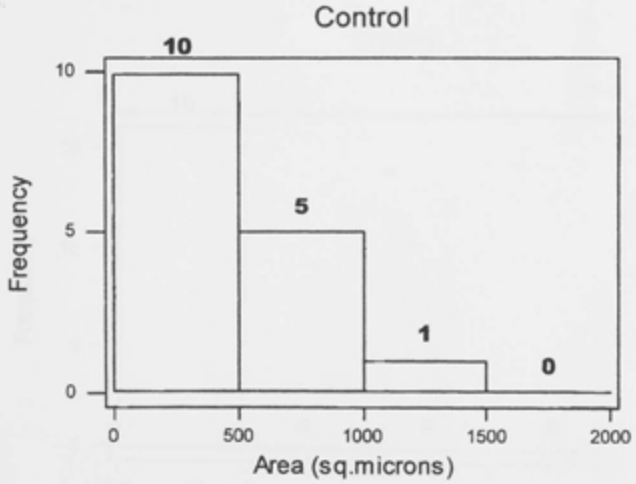


Fig-4.4: Frequency of occurrence of $CD8^+$ cells in the jejunum expressed as a percentage of total leukocyte population

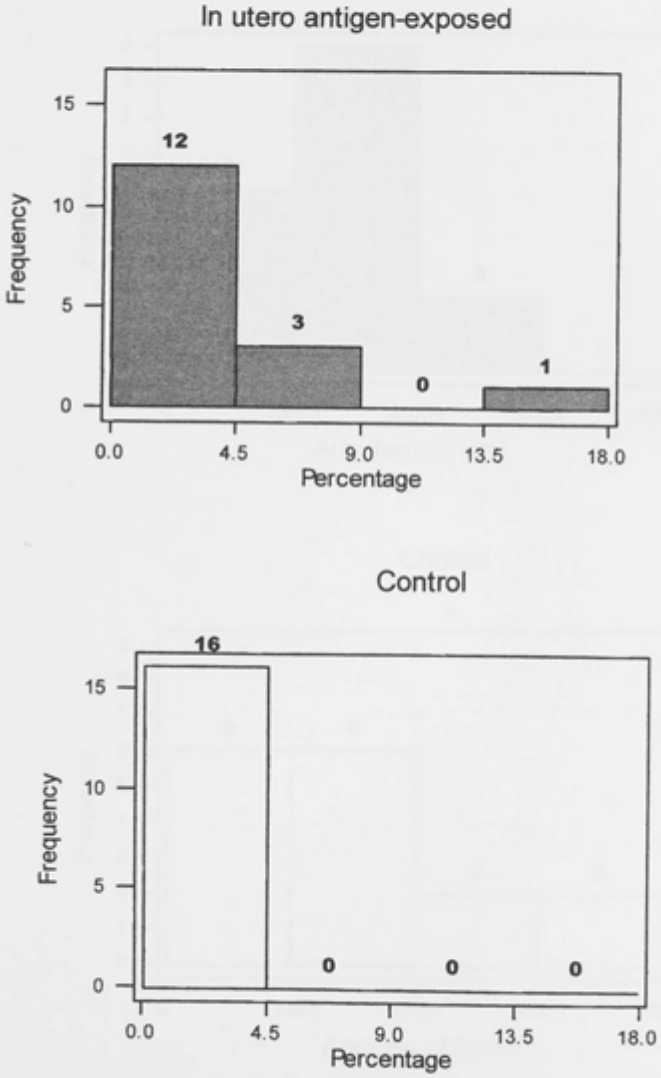
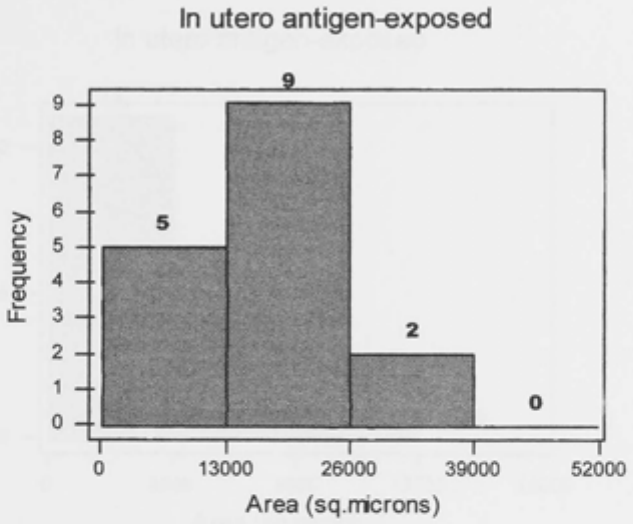


Fig-4.5: Frequency of occurrence of CD8$^+$cells in the MLN



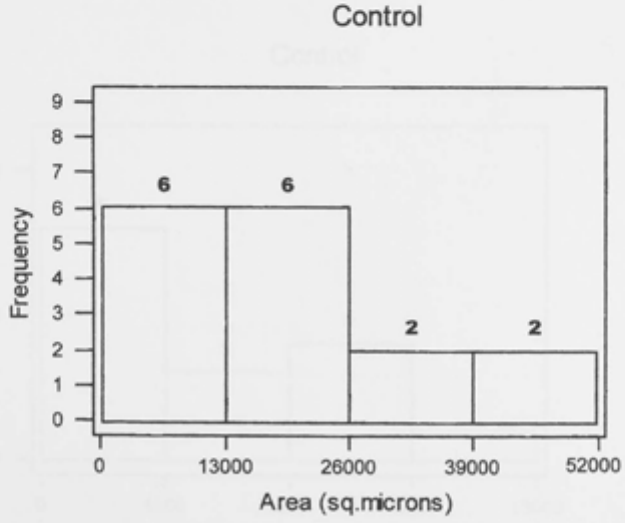
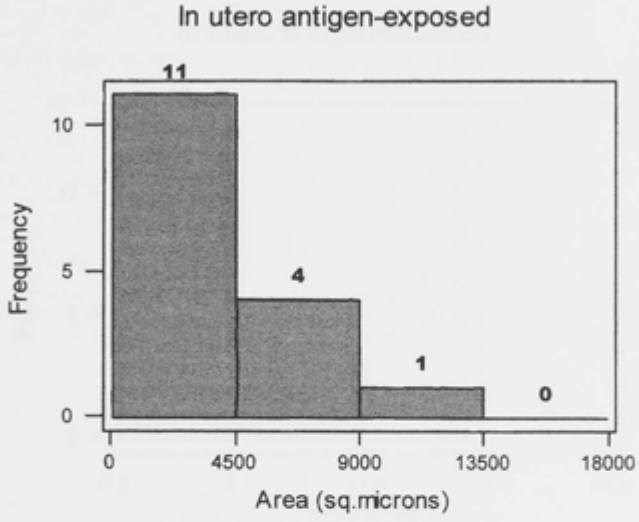


Fig-4.6: Frequency of occurrence of $CD8^+$cells in the IPP



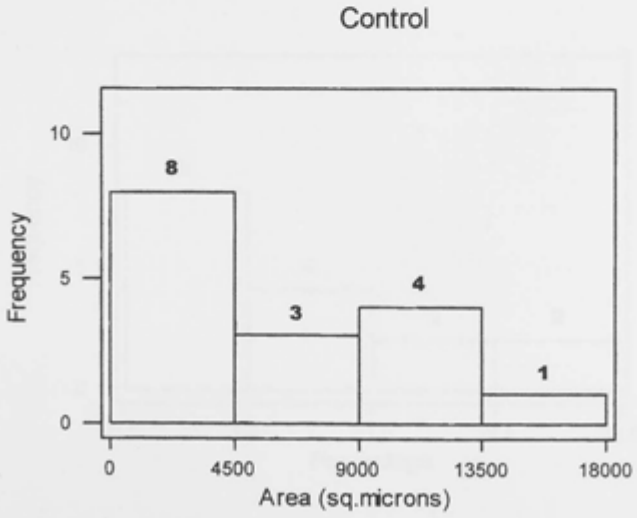


Fig- 4.7: Frequency of occurrence of $CD8^+$ cells in the IPP expressed as a percentage of total leukocyte population

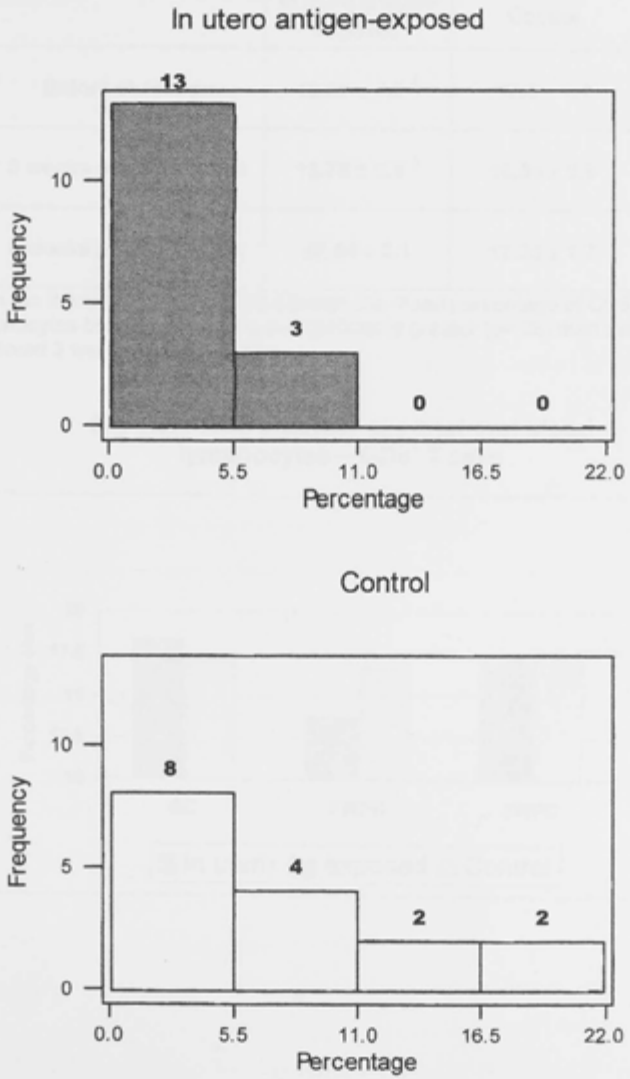
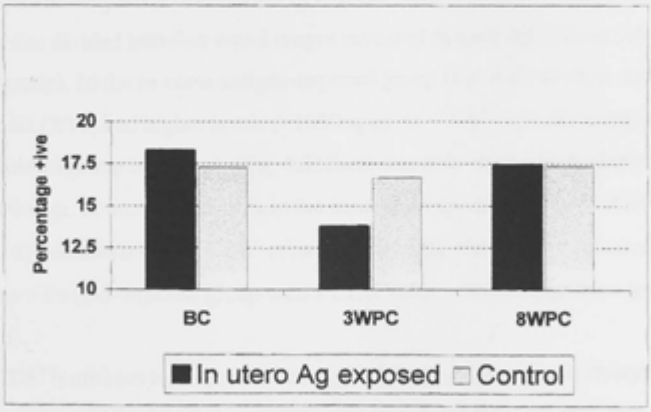


Table-4.8: FACS analysis of peripheral lymphocytes – $CD8^+$ T cells

Time	Mean ± S.E. (percentage)	
	In utero antigen-exposed	Control
Before challenge	18.28 ± 1.5 [A]	17.3 ± 1.4
3 weeks post-challenge	13.75 ± 2.3 [A]	16.69 ± 1.9
8 weeks post challenge	17.44 ± 2.1	17.35 ± 1.7

A: In the *in utero* antigen-exposed group, the mean percentage of $CD8^+$ lymphocytes before challenge was significantly greater (p=.05) than that observed 3 weeks post-challenge.

Fig-4.9: FACS analysis of peripheral blood lymphocytes – $CD8^+$ T cells



RESULTS

Jejunum :

Histology: Few histological differences were immediately apparent between the two groups of animals. In some animals, a number of $CD8^+$ cells could be seen clustered towards the luminal end of the mucosal villi. This tendency for clustering was more evident in the *in utero* antigen-exposed group of animals which had higher levels of $CD8^+$ lymphocytes in the jejunum. However, in most animals the positive cells were randomly distributed within the lamina propria singly or in clusters of a couple of cells (2-4 cells). There were a few positive intraepithelial lymphocytes and these stained very lightly. The $CD8^+$ cells found in the jejunum tended to be lighter staining than those found in the IPP and to have very hazy boundaries.

Image analysis:

a) Total positive area for $CD8^+$ lymphocytes: The mean area occupied by $CD8^+$ cells was significantly greater ($p= .048$) in the *in utero* antigen-exposed group (Table-4.1; Fig-A & B).

The frequency distribution of animals when the area occupied by $CD8^+$ cells was divided into four equal ranges revealed distinct differences between the two groups. In the *in utero* antigen-exposed group (Fig-4.3), a larger number of animals (7/16) had higher levels (>1000sq.μ) of $CD8^+$ cells. By comparison, in the control group of animals (Fig-4.3) there was only one animal with an area >1000 sq.μ. In accord with this in the control group, the majority of animals (15/16) had low levels of $CD8^+$ cells (<1000 sq.μ). The number of animals in the *in utero* antigen-exposed group with a $CD8^+$ area <1000 sq.μ were fewer (9/16).

b) $CD8^+$ lymphocytes expressed as a percentage of the total leukocyte population: The total $CD8^+$ positive area was divided by the total area staining with the leukocyte common antigen CD45 and the result expressed as percentage. The mean percentage of $CD8^+$ lymphocytes in the jejunum was significantly greater ($p=.032$) in the *in utero* antigen-exposed group of animals (Table-4.2).

Distinct differences between the two groups were observable in terms of frequency distribution of animals when the area of $CD8^+$ cells in the jejunum expressed as a percentage of the total leukocyte population, was divided into four

equal ranges. Whereas 75% of the animals (12/16) in the *in utero* antigen-exposed group had < 4.5 % of $CD8^+$ cells in the jejunum all the animals in the control group had < 4.5 % of $CD8^+$ cells (Fig-4.4). Expressed differently, in the *in utero* antigen-exposed group 25% of the animals (4/16) had > 4.5 % of $CD8^+$ lymphocytes while in the control group there were none.

Mesenteric lymph node:

Histology: Positive cells were clustered in patches between the trabeculae of the medullary region. As in the jejunum, positive cells tended to be lighter staining than those in the IPP in the majority of animals and the cell boundaries also tended to be hazy. However, in a few animals the positive lymphocytes tended to be darker staining with sharply defined borders.

Image analysis:

a) Total positive area for $CD8^+$ lymphocytes: The mean area occupied by $CD8^+$ lymphocytes was almost identical in the two groups (Table-4.1) .

The frequency distribution of animals when the area occupied by $CD8^+$ cells in the MLN was divided into four equal ranges revealed only marginal differences between the two groups (Fig-4.5). There were two animals with an area >39000 sq.μ in the control group of animals in comparison to the *in utero* antigen-exposed group where there were none.

b) $CD8^+$ lymphocytes expressed as a percentage of the total leukocyte population: The content of $CD8^+$ lymphocytes expressed as a percentage of the total leukocyte population were identical in the two groups (Table–4.2). There was no appreciable difference in the frequency distribution of the percentage of $CD8^+$ lymphocytes.

Ileal Peyer's patch:

Histology: There was no appreciable difference between the two groups of animals in the staining intensity or the distribution of $CD8^+$ lymphocytes within the IPP. Unlike the jejunum and the MLN, the $CD8^+$ cells were generally darker staining with sharp boundaries. This was especially noticeable among cells clustered within the interfollicular zone, which tended to be extremely dark staining with clear-cut boundaries. There were also a few positive cells within the mucosal villi, often clustered towards the luminal end. $CD8^+$ cells in mucosa were often found in close association with the B lymphocytes of the corona

region These cells included a mixture of dark and slightly lighter staining lymphocytes in contrast to the $CD8^+$ lymphocytes found in the interfollicular region. They were usually observed to be clustered in patches of 4-8 cells. A few (5-20) lymphocytes were also seen infiltrating into the follicles. In the *in utero* antigen-exposed group 9/16 animals had this histological feature while in the control it was observed in 10 lambs.

Image analysis:

a) Total area positive for $CD8^+$ lymphocytes: The mean area occupied by $CD8^+$ lymphocytes was significantly higher (p=.05) in the control group of animals (Table-4.1).

There were distinct differences between the two groups when the frequency distribution of area occupied by $CD8^+$ cells in the IPP was distributed into four equal ranges. In the *in utero* antigen-exposed group (Fig-4.6) there was only one animal with an area >9000 sq.μ in the IPP, while in the control group of animals there were five such animals (Fig-4.6).

b) $CD8^+$ lymphocytes expressed as a percentage of the total leukocyte population: $CD8^+$ lymphocytes expressed as a percentage of the total leukocyte population was significantly (p=.05) higher in the control group of animals in comparison with the *in utero* antigen-exposed lambs (Table-4.2).

The frequency distribution of animals when the area of $CD8^+$ cells in the IPP, expressed as a percentage of the total lymphocyte population, was divided into four ranges revealed distinct differences between the two groups. A majority of animals (13/16) in the *in utero* antigen-exposed group (Fig-4.7) had <5.5 % of $CD8^+$ cells within the IPP. In the control group 50% of animals (8/16) had low levels (<5.5 %) of $CD8^+$ cells (Fig-4.7). Additionally, in the *in utero* antigen-exposed group, only 3/16 animals had >5.5 % of $CD8^+$ cells. However, in the control group, 50% of the animals (8/16) had >5.5 % of $CD8^+$ cells.

FACS analysis of peripheral blood lymphocytes for $CD8^+$ cells: The mean levels of $CD8^+$ lymphocytes in the peripheral blood were not significantly different between the two groups at any of the three points of time (Table-4.8). The only appreciable difference was that, in the *in utero* antigen-exposed group of animals, three weeks post challenge the CD8 levels registered a significant (p=.05) drop from the pre-challenge levels (Table-4.8 and Fig-4.9) . This

significant drop in CD8 levels was not observed in the control group of animals three weeks after challenge with larvae.

DISCUSSION

There were some substantial differences between the *in utero* antigen-exposed and control lambs in the distribution of $CD8^+$ lymphocytes in the gastrointestinal tract and peripheral circulation. The $CD8^+$ levels, whether expressed as the total positive area or as the percentage of the total leukocyte population, were significantly higher in the jejunum of lambs that had been exposed to *Trichostrongylus colubriformis* third stage larval antigen *in utero*. Re-infection with certain parasites has been observed to induce an anamnestic immune response characterised by elevated levels of $CD8^+$ lymphocytes within the gastrointestinal tract. For example, in calves that were immune, re-infection with *Cryptosporidium parvum* resulted in an increase in the number of $CD8^+$ lymphocytes within the intestinal villi (Abrahamsen *et al.*, 1997). Likewise, McClure *et al.* (1992) have reported that, in *Trichostrongylus*-immune lambs an increased number of $CD8^+$ lymphocytes appeared in the epithelium and lamina propria of the jejunum on re-exposure to the same parasite. The results from the present study resemble this observation. However, the results of Gorrell *et al.* (1988b) are contrary to the observation of McClure *et al.* (1992) and to that of the present study. Gorrell *et al.* (1988b) observed that sheep (aged 30-34 weeks) which had been initially exposed to the live *Trichostrongylus* larvae and subsequently challenged with them did not manifest any changes in the $CD8^+$ lymphocyte population of the intestinal mucosa. Direct comparison of the results from the present study with those of Gorrell *et al.* (1988b) is unlikely to be feasible because of the differences in antigens and ages of animals in the two studies.

The observation that *in utero* antigen-exposed lambs had significantly higher levels of $CD8^+$ lymphocytes in the jejunum in comparison with control lambs demonstrates a very important point. It appears that the fetal lamb's gut immune system is sufficiently mature to be influenced by oral exposure to an antigen. The best indication of this is the substantial differences between *in utero* antigen-exposed and control lambs in the gut responses to *Trichostrongylus colubriformis*. The altered response observed in the *in utero* antigen-exposed

lambs could reflect the previous sensitisation of the lamb in fetal life with a resulting memory of that antigenic exposure. Three weeks after challenge with live larvae, there was a significant drop in the $CD8^+$ lymphocyte levels in the peripheral circulation of *in utero* antigen-exposed lambs which did not occur in the lambs that had not been previously been exposed to parasite antigen *in utero*. This could be interpreted as an indication that *in utero* antigen-exposed lymphocytes were being rapidly depleted from the peripheral circulation and being retained in the target site of infection, namely the gut. The occurrence of these changes 12 weeks after *in utero* antigenic exposure suggests long term induction of memory to the parasitic antigen. This apparent *in utero* sensitisation implies that the foetal gut mucosal immune system 50 days before birth may not be markedly different from that of the post-natal lamb.

The question that arises in this context concerns the nature of mechanisms involved in the amplification of the $CD8^+$ lymphocyte response in the jejunum of *in utero* antigen-exposed lambs. During response to re-exposure, the population of $CD8^+$ T lymphocytes could have been expanded locally (Bloom & Boedeker, 1996) although evidence to support this is lacking in this study. It is also possible that there could be re-population of lymphocytes from the IPP (Bloom & Boedeker, 1996). There is evidence to support this possibility in the present study. After larval challenge, the $CD8^+$ T lymphocyte levels, expressed either as total positive area or as percentage of total lymphocytes were significantly higher in the IPP of control lambs in comparison with these values in the *in utero* antigen-exposed lambs. These observations suggest that considerable numbers of $CD8^+$ T lymphocytes were retained in the IPP of control animals despite the ongoing infection in the jejunum. The $CD8^+$ T lymphocytes in the IPP of *in utero* antigen-exposed lambs seem to be preferentially homing to the target site of infection, the jejunum, resulting in low levels of $CD8^+$ lymphocytes in the IPP.

It is evident from the present study that $CD8^+$ T lymphocyte levels at different anatomical sites in the gastrointestinal tract differ considerably in the two groups of animals. Among *in utero* antigen-exposed lambs, when the level of $CD8^+$ T lymphocytes in the jejunum was significantly higher the corresponding level in the IPP was significantly lower in comparison with that observed in the jejunum and IPP of control animals respectively. Gorrell *et al.* (1988b) have also

observed a divergent $CD8^+$ T lymphocytes response in different gastrointestinal tract sites following *T. colubriformis* infection of naive lambs. They observed a marked reduction in the number of $CD8^+$ T cells in the lamina propria of the duodenal tissue taken within one metre from the pylorus. In contrast, jejunal tissue collected five metres distal to the pylorus did not manifest any change in the number of $CD8^+$ T cells four weeks after challenge . It was also evident from the present study that at most times, the $CD8^+$ T lymphocyte levels in the peripheral circulation did not reflect the divergent levels of $CD8^+$ T lymphocytes in the gastrointestinal tract. These features clearly suggest that the dynamics of the $CD8^+$ T lymphocyte response in different regions of the gastrointestinal tract is distinctly different. Thus any study that draws conclusions about what $CD8^+$ T lymphocytes levels do without undertaking a proper focus on different regions of the gastrointestinal tract should be interpreted with caution.

The actual functional attributes of the $CD8^+$ T lymphocytes in the gastrointestinal tract of infected lambs were not assessed in the present study. However, it appears that the increased levels of $CD8^+$ T lymphocytes in the jejunum may be actually down-regulating the immune response in the gastrointestinal tract of *in utero* exposed lambs. The marginally higher worm count in the small intestine of *in utero* antigen-exposed lambs might reflect this. The observations of McClure *et al.* (1996) are consistent with the interpretation that a stronger $CD8^+$ T lymphocytes response in the gut of *Trichostrongylus*-infected animals was associated with a less vigorous, down-regulated protective response in the gut.

McClure *et al.* (1992) have reported that following challenge of immune sheep with the third stage larvae of *T. colubriformis* there was increased infiltration of $CD8^+$ lymphocytes into the lamina propria and the mucosal epithelium of the jejunum. However, this increase of $CD8^+$ cells persisted for only five days post challenge and then declined back to the low pre-challenge levels. In contrast, the present study observed that significantly elevated levels of $CD8^+$ cells could be detected in the jejunum of *in utero* antigen-exposed lambs eight weeks after larval challenge, in comparison with control lambs. Thus, in lambs *in utero* oral antigenic exposure followed by re-exposure to the same antigen in postnatal life seems to induce a prolonged infiltration of $CD8^+$

lymphocytes into the gut. A significant proportion of the $CD8^+$ cells found in the lamina propria of the gut is presumed to represent suppressor/ regulatory T cells (McGowan *et al.*, 1997). The inflammatory response in the gut of *in utero* antigen-exposed lambs after challenge seems to be considerably higher and to be manifested clinically as diarrhoea and immunohistologically as increased levels of pro-inflammatory cytokines like TNF-α in the jejunum and MLN. It could be speculated that these infiltrating $CD8^+$ T cells might be down-regulating the increased inflammatory responses which, if left unchecked, might be detrimental to the host.

Another feature consistently observed in the present study was the variation between different anatomical sites of the gastrointestinal tract in the intensity of staining of $CD8^+$ T lymphocytes. This variation was also observed in the case of NSE positive cells in the gut. No clear-cut explanation for this difference is evident from the published literature. Perhaps, the lymphocytes staining more darkly within the IPP could be the ones that are actively undergoing induction to initiate an immune response. The lighter staining lymphocytes within the jejunum and MLN could then be cells that are in the process of carrying out their effector functions or have already completed them. However, this explanation remains speculative.

One of the important criteria for identifying the IPP as a primary lymphoid organ has been the low level of $CD8^+$ T lymphocytes reported in the tissue. Studies by Hein *et al.* (1989) and Aleksandersen *et al.*(1990) have consistently reported that $CD8^+$ T lymphocytes within the IPP represent less than 1% of the total lymphocyte population. However, the present study did not find any evidence to support their observations. Even in the *in utero* antigen-exposed lambs which had very low levels of $CD8^+$ lymphocytes in the IPP the mean level was 3.8 ± 0.6 %. In control lambs the percentage of $CD8^+$ T lymphocytes in the IPP was almost twice (6.6 ± 1.5 %) that observed among *in utero* antigen-exposed lambs. This difference could be on account of the fact that both these workers (Aleksandersen *et al.*, 1990; Hein *et al.*, 1989) carried out FACS analysis of the dissociated IPP. Furthermore, their inferences were based on investigations with a very limited number of uninfected animals. Alternatively, there may be a breed difference in $CD8^+$ T lymphocyte content of IPP.

The presence of a few $CD8^+$ lymphocytes infiltrating into the follicles of the IPP suggest the possibility that T cells may be involved in the primary B cell development or that the IPP can have some secondary function during parasitic infections.

CD5

INTRODUCTION

The CD5 molecule is a 67 KDa surface glycoprotein which is expressed on the majority of T lymphocytes as well as on the B-1 subsets of B lymphocytes (Mackay, 1988; Youinou *et al.*, 1999). CD5 belongs to the scavenger receptor cysteine-rich domain superfamily (Bauch *et al.*, 1998). The CD5 molecule on lymphocytes is functionally and physically associated with the antigen receptor (Bauch *et al.*, 1998; Birkebak *et al.*, 1994). Its precise function has not been determined. However, it is believed that the CD5 molecule is involved in signal transduction and in B cell/ T cell interactions (Birkebak *et al.*, 1994).

Resting lymphoid populations of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells all express CD5 (Mackay, 1988). Proliferating immature, cortical thymocytes also express CD5. However, in the sheep, activated T cells no longer express CD5 and, as a consequence, there are large proportions of $CD4^+$ $CD5^-$ and $CD8^+$ $CD5^-$ cells in efferent lymph after *in vivo* activation of the lymph node (Hopkins & Dutia, 1990). Furthermore, the CD5 marker is also lost after *in vitro* stimulation of peripheral blood mononuclear cells with conA (Hopkins & Dutia, 1990). This may explain why the total numbers of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ cells (Mackay *et al.*, 1985) in the peripheral blood of Border disease infected sheep (Burrells *et al.*, 1989), or in lesions produced by the parasites *Haemonchus* (Gorrell *et al.*, 1988a) and *Trichostrongylus* (Gorrell *et al.*, 1988b), far exceed the numbers of $CD5^+$ lymphocytes.

The finding that the T cell marker CD5 was expressed by a small proportion of normal B cells led to the concept that there are at least two main subpopulations of B lymphocytes. The B-1 cells encompass the $CD5^+$ subpopulation, whereas B-2 cells represent the conventional B cell subpopulation (Youinou *et al.*, 1999). The most significant feature of B-1 cells is their production of low affinity, polyreactive immunoglobulins. It is thought that B-1 cells might play an important role in maintaining homeostasis in the gut. IgA secreting cells have a dual origin and are derived either from conventional B cells or from B-1 cells. The major antigenic targets of B-1 cell-derived IgA secreting cells are normal intestinal bacteria. The fixed and biased repertoire of B-1 cells might play a role in maintaining the normal balance of the intestinal flora. When

pathogenic bacteria penetrate into the gut, conventional B cells in the Peyer's patches may be induced to produce high affinity IgA antibodies leading to immune exclusion (Kroese *et al.*, 1996). $CD5^+$ B lymphocyte populations are increased in size in some autoimmune diseases and leukaemias (Youinou *et al.*, 1999). Classical B-1 cells appear to be mainly T cell independent, although they are positively influenced by T cell derived cytokines (Youinou *et al.*, 1999).

Several investigators have carried out the quantitative characterisation of the $CD5^+$ lymphocyte population in the peripheral blood of normal sheep. Keech and Brandon (1991a) have reported that 72-76 % of peripheral blood lymphocytes express CD5. However, this observation was based on investigation of a very limited number of animals. Birkebak *et al.* (1994) also characterised the $CD5^+$ lymphocytes in the peripheral blood of normal sheep using a larger number of animals. They observed that significant variation in $CD5^+$ lymphocyte numbers occurred in all animals over the time span of 10 weeks in which they undertook the study. The total $CD5^+$ cell population comprised 31- 65.4 % of the gated lymphocytes with a mean value of 44.9 %. Birkebak *et al.* (1994) also calculated that the mean ratio of $CD5^+$ B lymphocytes to the total B lymphocyte population was 0.1 with a standard deviation of 0.029.

Pernthaner *et al.*(1996) evaluated the immune responsiveness of nematode-resistant and susceptible lines of Romney sheep to a continuous infection with *Trichostrongylus axei*. Significant increases in the proportions of $CD5^+$, $CD4^+$, $CD8^+$ and $\gamma\delta^+$ cells occurred in both resistant and susceptible line lambs during the first four weeks of infection. Following peak levels, the proportions of $CD5^+$, $CD4^+$ and $CD8^+$ cells fell with the rate of decline of $CD5^+$ and $CD4^+$ T cells being significantly greater in the resistant line lambs. Pernthaner *et al.* (1996) also observed that susceptible line lambs possessed higher proportions of $CD5^+$ and lower proportions of B cells than resistant lambs before infection with the parasite.

Age is an important factor determining the $CD5^+$ lymphocyte response in the peripheral circulation of *Trichostrongylus colubriformis* infected sheep. Kambara and McFarlane (1996) have shown that repeated immunisation with the larvae of *T. colubriformis* over 12 weeks, resulted in distinctly different $CD5^+$ lymphocyte responses in the peripheral circulation of both younger (8-26

weeks) and older (31-51 weeks) animals. There was an increase in the percentage of $CD5^+$ lymphocytes in the peripheral blood of older animals whereas in the younger animals the levels of these cells decreased. The increase in $CD5^+$ cells observed in the older animals was predominantly attributable to the increase in $CD4^+$ lymphocytes, and to a limited extent, to changes in $CD8^+$ and $\gamma\delta^+$ T cells.

The ontogeny of leukocyte populations in the IPP of sheep was investigated by Press *et al.* (1992). In the foetal lamb at 70 days of gestation, $CD5^+$ lymphocytes can be detected in the lamina propria and along the line of the muscularis mucosae of the gut. Although large numbers of $CD5^+$ lymphocytes predominated in this tissues at this stage of gestation, only a small number of $CD4^+$ and $CD8^+$ lymphocytes could be simultaneously detected. After 100 days of gestation there was a steady expansion in the number of $CD5^+$ cells. Beyond 115 days of gestation, the anatomical distribution of $CD5^+$ lymphocytes was identical with that seen in the IPP of the postnatal lamb (Press *et al.*, 1992).

Gorrell *et al.* (1988b) have reported that, in the normal sheep gut, less than 1 % of the intraepithelial lymphocytes express CD5. In contrast, Gyorffy *et al.* (1992) found a higher percentage of $CD5^+$ lymphocytes within the intraepithelial lymphocytes and the lamina propria of the sheep gut. They observed that, among the intraepithelial lymphocytes, 52 % expressed CD5. A notable feature of intraepithelial lymphocytes in sheep was the presence of a distinct $CD8^+$ and $\gamma\delta^+$ populations that lacked CD5. Gorrell *et al.* (1988b) have suggested that those circulating $CD8^+$ and $\gamma\delta^+$ cells that localise in the gut might become activated and cease to express CD5 under the influence of the local microenvironment. The percentage of $CD5^+$ lymphocytes within the lamina propria was considerably higher than that among intraepithelial lymphocytes with 69% of the lamina propria lymphocytes expressing CD5.

There is considerable variation in the expression of CD5 within the different anatomical sites of the gastrointestinal tract. Aleksandersen *et al.* (1990) observed that an average 25 % of the lymphocytes from the large intestine lymphoid patches and the jejunal Peyer's patches expressed CD5. In contrast, less than 1 % of the lymphocytes from the ileal Peyer's patches expressed CD5. Hein *et al.* (1989) also observed distinct difference in the expression of CD5 within the jejunal Peyer's patches and the ileal Peyer's patches. The lymphocyte suspension

Table- 5.1: Total positive area – $CD5^+$ cells

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	3297 ± 652	3254 ± 548	NS
Mesenteric lymph node	25762 ± 4624	25162 ± 3377	NS
Ileal Peyer's patch	6580 ± 689	12767 ± 3206	P=.039

Table- 5.2: Relationship between the total T cells population and CD5 expression

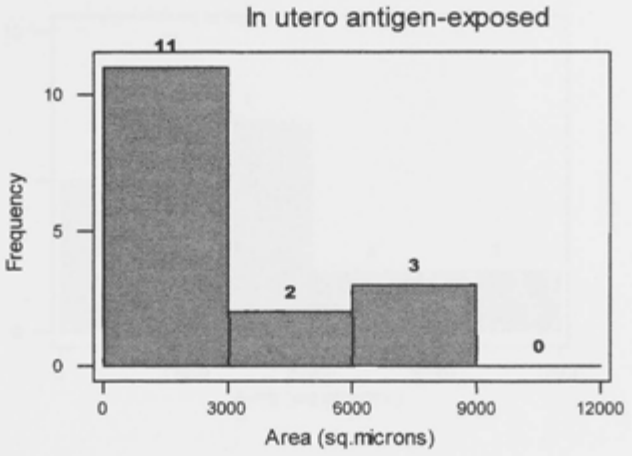
Tissue	Group	Mean ± S.E. (sq.μ)		Ratio of Total T cell: Total $CD5^+$cells
		Total area** - $CD4^+$,$CD8^+$ and γ δ T cells (Total T cells)	Total area – $CD5^+$cells	
Jejunum	*In utero* antigen-exposed	4969 ± 431	3254 ± 548	2.02 ± 0.24
	Control	5212 ± 593	3297 ± 652	1.91 ± 0.17
Mesenteric lymph node	*In utero* antigen-exposed	33505 ± 2303	25762 ± 4624	2.79 ± 0.89
	Control	37510 ± 5529	25162 ± 3377	1.74 ± 0.22
Ileal Peyer's patch	*In utero* antigen-exposed	11155 ± 1044[A]	6580 ± 689[B]	1.88 ± 0.22
	Control	17572 ± 2771[A]	12767 ± 3206[B]	1.87 ± 0.19

A: The total T cell population was significantly greater (p=.022) in the IPP of control animals in comparison with *in utero* antigen-exposed lambs.
B: The $CD5^+$ area in the IPP of control lambs was significantly greater (p=.039) in the control group in comparison with *in utero* antigen-exposed lambs
** The total area positive for $CD4^+$ and $CD8^+$ cells were obtained from Tables 3.1 and 4.1 respectively. The total area positive for γδ is furnished in table 5.3.

Table- 5.3 : Total positive area – γ δ (MAb CC15)

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	2110.9 ± 254.9	2577.6 ± 423.1	NS
Mesenteric lymph node	4040.8 ± 607.9	4061 ± 693.9	NS
Ileal Peyer's patch	3593.3 ± 457.2	3903.3 ± 439.2	NS

Fig-5.4: Frequency of occurrence of $CD5^+$ cells in the jejunum



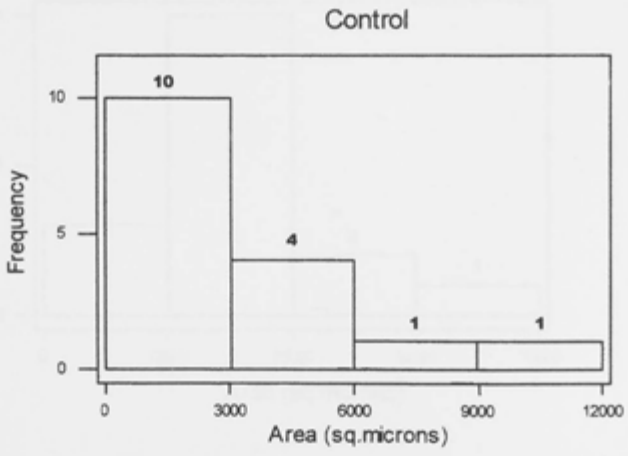


Fig-5.5: Frequency of occurrence of $CD5^+$ cells in the MLN

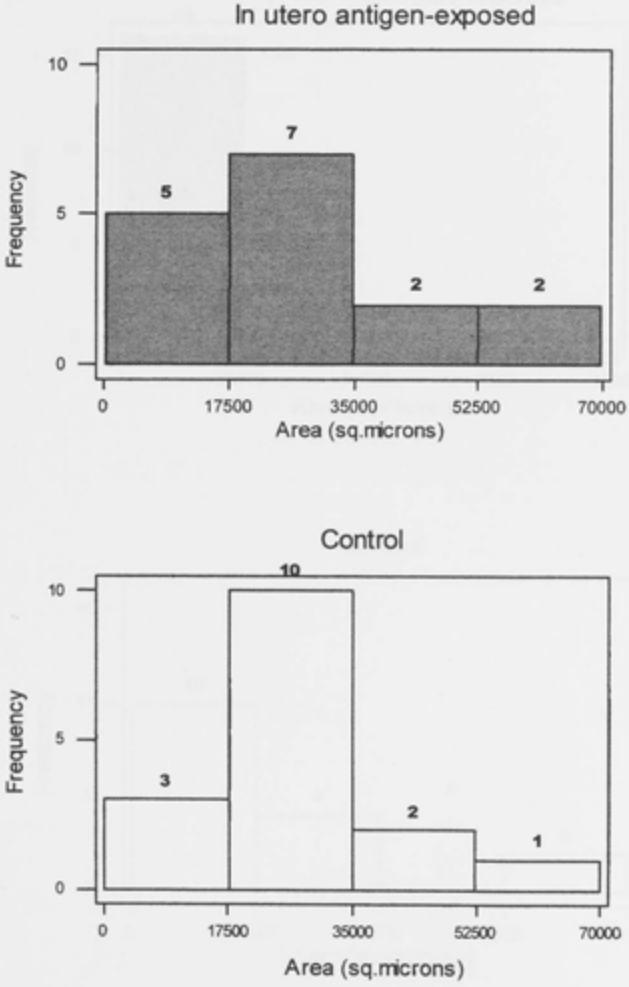
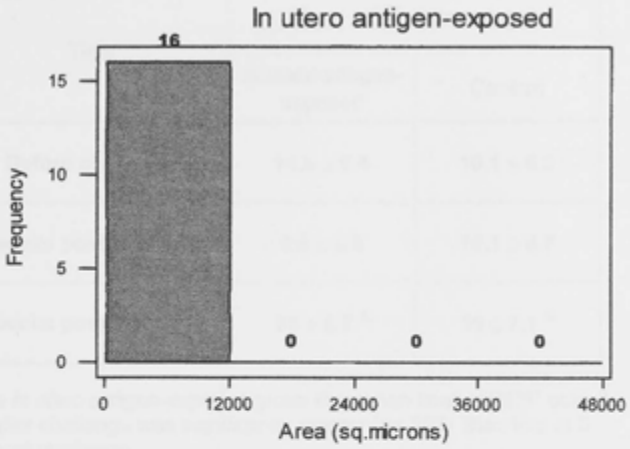


Fig-5.6: Frequency of occurrence of $CD5^+$ cells in the IPP



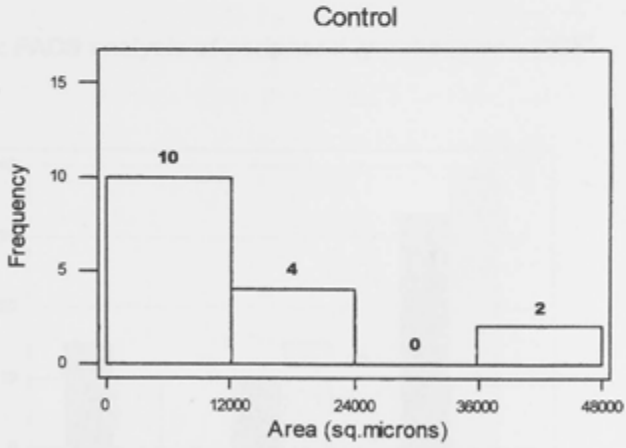
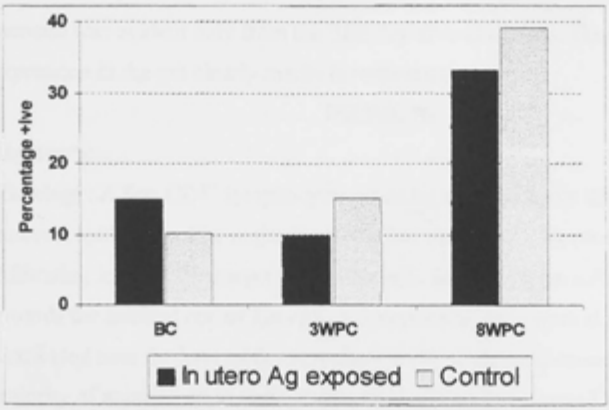


Table-5.7: FACS analysis of peripheral lymphocytes – $CD5^+$ cells

Time	Mean ± S.E. (percentage)	
	In utero antigen-exposed	Control
Before challenge	14.9 ± 6.4	10.1 ± 6.2
3 weeks post-challenge	9.6 ± 5.6	15.1 ± 5.7
8 weeks post-challenge	33 ± 6.7 [A]	39 ± 7.1 [B]

A: In the *in utero* antigen-exposed group the mean level of $CD5^+$ cells 8 weeks after challenge was significantly greater (p=.005) than that at 3 weeks post challenge.
B: In the control group the mean level of $CD5^+$ cells 8 weeks after challenge was significantly greater (p=.007) than that at 3 weeks post challenge.

Fig- 5.8: FACS analysis of peripheral lymphocytes – $CD5^+$ cells



prepared from the jejunal Peyer's patches included 16 % lymphocytes staining for the CD5 marker. Nearly all of the $CD5^+$ lymphocytes were also $CD4^+$ T cells (14 %), whereas only 2 % and <1 % respectively expressed the CD8 and γδ T cell markers. In contrast, Hein *et al.* (1989) observed that the ileal Peyer's patches contained <1 % of $CD5^+$ cells.

$CD5^+$ B cells have also been demonstrated within the gut of sheep. In the ileal Peyer's patches they comprise 2 % of the total B cell population. The mesenteric lymph nodes contain a higher percentage of B-1 cells with 6 % of the total B cell population expressing the CD5 marker (Chevallier *et al.*, 1998).

CD5 expression by lymphocytes within the gut of nematode infected sheep has also been investigated with conflicting outcomes. Sheep infected with <20,000 larvae of *Trichostrongylus colubriformis* larvae have been reported not to manifest any changes in the number of $CD5^+$ lymphocytes within the gut (Gorrell *et al.*, 1988b). In contrast, McClure *et al.* (1992) have observed that *T. colubriformis* infection altered the number of $CD5^+$ lymphocytes in the gut. Among naïve animals there was a slight increase in the number of $CD5^+$ cells three days after the onset of infection. CD5 expression remained elevated but did not increase further for the next 14 days of the study. However, among immune animals, *T. colubriformis* larval challenge was followed by a much greater increase in the number of $CD5^+$ lymphocytes within the lamina propria. This increase was evident only from the fifth day after challenge. The role of CD5 expression in the gut clearly merits investigation.

RESULTS

Jejunum:

Histology: A few $CD5^+$ lymphocytes could be seen randomly distributed in the mucosal epithelium. The majority of positive cells were observed to be infiltrating into the lamina propria of the villi. Some of these cells were clustered towards the luminal end of the villi. A few positive cells were also seen randomly distributed near the base of the villi close to the muscularis mucosae. In a majority of animals, from both groups, the positive cells stained with high intensity. Occasionally, a few animals had $CD5^+$ lymphocytes that stained with very low intensity. No histological differences were apparent between the two groups .

Image analysis: The mean area stained by $CD5^+$ lymphocytes was almost identical in the two groups (Table-5.1).

When the area stained by the $CD5^+$ cells in the jejunum was divided into four equal ranges and correlated with the frequency distribution of animals very little difference was apparent between the two groups (Fig-5.4).

Relationship between total T cell population and CD5 expression: The total T cell population was calculated by taking the sum of the area stained by $CD4^+$, $CD8^+$ and $\gamma\delta^+$ (Monoclonal antibody CC15) T cells {The data obtained when tissues were examined for the presence of $\gamma\delta$ is presented in table 5.3}. The ratio of the total T cell positive area to the total $CD5^+$ area was almost identical in the *in utero* antigen-exposed and the control lambs (Table-5.2).

Mesenteric lymph node:

Histology: The monoclonal antibody SBU T1 tended to stain most of the T cell areas as well as some scattered individual cells in the B cell zones. There appeared to be distinct differences between the two groups of lambs in the intensity of staining. The majority of animals (10/16) from the *in utero* antigen-exposed lambs had lymphocytes that stained with very high intensity. In contrast, only 5/16 lambs from the control group had MLN lymphocytes that stained with very high intensity, lymphocytes of the majority of animals (11/16) stained with a low intensity for CD5.

Image analysis: The mean area stained by the monoclonal antibody against $CD5^+$ lymphocytes was almost identical in the two groups of lambs (Table-5.1).

When the area stained for $CD5^+$ cells in the MLN was divided into four equal ranges and correlated with the frequency distribution of animals, only marginal differences were apparent between the two groups. In the *in utero* antigen-exposed lambs there were only 7/16 animals with a positive area >17500 and <35000 sq.μ while in the control group there were 10/16 lambs which fell within the same range (Fig-5.5).

Relationship between total T cell population and CD5 expression: Although not statistically significant, the ratio of total T cell positive area to the total $CD5^+$ area was higher in the *in utero* antigen-exposed lambs in comparison with control lambs (Table-5.2).

Ileal Peyer's patch:

Histology: The majority of the $CD5^+$ lymphocytes were distributed in the interfollicular region and within the mucosal villi. Very often the cells within the villi were seen clustered towards the luminal end of the villi. In some animals $CD5^+$ lymphocytes were also seen within the dome region of the follicles. Likewise, in some animals a few $CD5^+$ cells could be detected completely inside the follicles. The $CD5^+$ cells stained with high intensity in the majority of animals from both groups of lambs. In the *in utero* antigen-exposed lambs there were fewer $CD5^+$ lymphocytes within the IPP. In contrast, among the control lambs there were substantially more $CD5^+$ cells within the IPP sections.

Image analysis: The mean area stained by $CD5^+$ cells was significantly greater ($p=.039$) in the control group of lambs in comparison with the *in utero* antigen-exposed lambs (Table-5.1).

The frequency distribution of animals when the area occupied by $CD5^+$ cells in the IPP was divided into four equal ranges revealed distinct differences between the two groups. In the *in utero* antigen-exposed group, all the animals (Fig-5.6) had a $CD5^+$ area <12000 sq.μ. In contrast, only 10/16 animals from the control group (Fig-5.6) had a $CD5^+$ area <12000 sq.μ. Six lambs from the control group (Fig-5.6) had high levels of $CD5^+$ area (>12000 sq.μ) while there were none in the *in utero* antigen-exposed group.

Relationship between total T cell population and CD5 expression: The ratio of the total T cell positive area to total $CD5^+$ area was almost identical in the two groups of lambs (Table-5.2).

$CD5^+$ cells expressed as a percentage of the total leukocyte population: The total $CD5^+$ positive area was divided by the total area stained by the leukocyte common antigen marker CD45 and then expressed as a percentage. The percentage of $CD5^+$ leukocytes in the IPP was significantly greater ($p=.032$) in the control group (12 ± 2.9 %) in comparison with the *in utero* antigen-exposed lambs (6.15 ± 0.7 %).

FACS analysis of peripheral blood: Prior to challenge, the percentage of $CD5^+$ lymphocytes in the peripheral blood was higher in the *in utero* antigen-exposed lambs in comparison with the control lambs (Table-5.7 and Fig-5.8). Three weeks after the live larval challenge, there was a drop in the percentage of

$CD5^+$ lymphocytes in the peripheral blood of *in utero* antigen-exposed lambs (Table-5.7 and Fig-5.8). In contrast, among control lambs the $CD5^+$ levels increased three weeks after the larval challenge (Table-5.7 and Fig-5.8). Both groups of lambs had significantly elevated levels of $CD5^+$ lymphocytes in the peripheral circulation eight weeks after challenge. At this time the percentage levels of $CD5^+$ lymphocytes in peripheral blood tended to be marginally higher in the control group of lambs (Table-5.7 and Fig-5.8).

DISCUSSION

The CD5 antigen is present on the surface of all T lymphocytes and a low proportion of B lymphocytes (Mackay, 1988; Youinou *et al*., 1999). The most significant differences between the two experimental groups of lambs in the expression of the CD5 antigen was evident in the IPP. The majority of the ileal $CD5^+$ lymphocytes were distributed in the interfollicular region and in the lamina propria of the villi. A number of animals also had positive cells within the follicles and in the dome region of the follicle. This contrasts with CD5 expression within the IPP of uninfected sheep which has been reported to be almost exclusively confined to the interfollicular region (Aleksandersen *et al*., 1990; Hein *et al*., 1989). It is possible that the parasitic infection in the present experiment resulted in a more extensive expression of the CD5 antigens by lymphocytes of the IPP.

The CD5 levels, whether expressed as the total positive area or as the percentage of the total leukocyte population were significantly higher in the IPP of control lambs than in the *in utero* antigen-exposed lambs. The expression of the CD5 molecule on lymphocytes is unusual in that it is differentially expressed on resting and activated cells. The CD5 molecule is lost from sheep lymphocytes on cellular activation (Hopkins & Dutia, 1990). If this evidence is taken into account it gives an impression that the IPP from the *in utero* antigen-exposed lambs contained increased numbers of activated lymphocytes because of the significantly reduced expression of CD5 by lymphocytes. However, when other relevant data was also taken into account it became clear that this might not be the case.

The vast majority of $CD5^+$ lymphocytes detected within the IPP are T cells since only 2 % of the total B cell population are B-1 lymphocytes

(Chevallier *et al.*, 1998). The total T cell positive area as calculated based on the sum of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ cells was significantly different between the two group of lambs. The control group of lambs had a significantly larger total T cell positive area in the IPP in comparison with the *in utero* antigen-exposed lambs. The ratio of the total T cell positive area to the $CD5^+$ area was almost identical in the two groups of lambs. This was an indication that the proportion of T cells expressing the CD5 marker in relation to the total T cell population was almost identical in the two groups of animals. The significant difference in the expression of CD5 antigen within the IPP of the two groups of lambs was attributable to the difference in the total T cell population. The IPP of control lambs had significantly higher levels of $CD4^+$ and $CD8^+$ T lymphocytes while the $\gamma\delta^+$ T cell population in the tissues of these animals was also marginally higher in comparison with their respective levels in the *in utero* antigen-exposed lambs. Thus, *in utero* antigenic exposure followed by re-exposure to homologous antigen in postnatal life was able to bring about a significant alteration in CD5 expression within the IPP. However, this significant change seems to have been brought about by the marked depletion of $CD4^+$ and $CD8^+$ T lymphocytes from the IPP rather than by increased activation of the lymphocytes.

Studies by Hein *et al.* (1989) and Aleksandersen *et al.* (1990) have consistently reported that in the IPP of uninfected sheep <1 % of the total lymphocytes population express the CD5 marker. The present study produced findings that were substantially different from these earlier observations. Even in the *in utero* antigen-exposed lambs which had significantly lower levels of expression of the CD5 antigen in the IPP the mean level of $CD5^+$ lymphocytes was 6.15 ± 0.7 %. In control lambs the percentage of $CD5^+$ lymphocytes in the IPP was 12 ± 2.9 % which was twice the number detected in the lambs exposed *in utero*. This difference from the earlier reports could reflect the fact that both of these groups of workers (Aleksandersen *et al.*, 1990; Hein *et al.*, 1989) carried out FACS analysis of the dissociated IPP whereas the present study was undertaken on intact tissue. An important difference of protocol which may explain the differing results was that their conclusions was based on the investigation of a limited number of uninfected animals whereas all the lambs

examined in the present study had been infected. There may also be differences between sheep breed strain (Merino versus European strains).

The expression of CD5 within the MLN was unique in certain aspects. The majority of lambs from the *in utero* antigen-exposed group had MLN lymphocytes that stained with a high intensity for CD5. In contrast, the lymphocytes from the MLN of control lambs stained with low intensity. Activation of lymphocytes is associated with a loss of CD5 expression (Hopkins & Dutia, 1990). The reduced expression of CD5 antigen by the lymphocytes of the MLN from control animals suggests that these cells might have be passing though a phase of activation. The substantially higher levels of IL-2, a potent activator of lymphocytes (Smith, 1992), within the MLN of control lambs in comparison with *in utero* antigen-exposed animals could have resulted in the down-regulation of CD5 expression. The darker staining lymphocytes in the MLN of *in utero* antigen-exposed lambs could be an indication that they had not been activated to the same extent as the cells of the control lambs. The low levels of IL-2 within the MLN of *in utero* antigen-exposed lambs could have resulted in reduced activation of lymphocytes resulting in marked expression of CD5.

In the MLN, the ratio of the total T cell positive area to the $CD5^+$ area was higher (but not significant statistically) in the *in utero* antigen-exposed group in comparison with the control lambs. Of the three tissues examined, the MLN was the only tissue in which the ratio of total T cell population to the $CD5^+$ area was different between the *in utero* antigen-exposed and control lambs. This difference could have arisen as a result of the different extent of activation of the T lymphocytes in the different tissues with a resulting loss of CD5 expression. There is a possibility that $CD5^+$ expressing B cells might have also played a role in altering this ratio. The MLN of sheep have been reported to contain a higher percentage of CD5 expressing B cells than the IPP with 6 % of the total B cell population expressing this marker (Chevallier *et al.*, 1998).

In the jejunum, the mean area staining for CD5 was almost identical in the two groups of lambs. The expression of CD5 in the jejunum was not altered by the *in utero* antigenic exposure. Gorrell *et al.* (1988b) have also observed that *Trichostrongylus colubriformis* infected sheep did not manifest any increase in the number of $CD5^+$ lymphocytes within the gut. However, the results of the

present study differs from the findings of McClure *et al.* (1992) who observed that both naïve and *T. colubriformis* immune sheep manifested increased numbers of $CD5^+$ lymphocytes within the gut after challenge with the parasite. They also observed that the magnitude of the response was higher in immune animals compared with naïve animals. Both the age of the lambs used and the nature and the dose of antigen used for sensitisation in that study were distinctly different from those of the present study and this could have accounted for the different CD5 response.

The percentage of $CD5^+$ lymphocytes detected in the peripheral blood of lambs before challenge was much lower that that reported in the literature. The *in utero* antigen-exposed lambs had a mean level of 14.9 % while control lambs had 10.1 % of $CD5^+$ peripheral lymphocytes. Keech and Brandon (1991a) used a limited number of animals in their study and reported that 72 – 76 % of the peripheral blood lymphocytes express CD5. Birkebak *et al.* (1994) examined the peripheral blood of sheep aged 9-12 months and observed that, on average 44.9 % of the gated lymphocytes were $CD5^+$. In the present study, the average age of the lambs before larval challenge was 4 weeks. The very young age of the lambs could have been the reason for the detection of very low levels of $CD5^+$ lymphocytes in the peripheral blood. Towards the end of the study when the average age of the lambs was three months, $CD5^+$ lymphocyte levels in the peripheral blood had risen and almost reached adult levels. The reason for the low CD5 cell numbers were thought to be age-related and is also supported by the report of Kambara and McFarlane (1996). They used the same monoclonal antibody marker on 3 month old lambs and observed that 30-50 % of the peripheral blood lymphocytes were $CD5^+$. In contrast, at 10 months of age, lambs had 60-80 % of their peripheral lymphocytes expressing CD5 irrespective of challenge.

Before challenge with the live third stage larvae, the *in utero* antigen-exposed lambs had higher levels of $CD5^+$ lymphocytes in the peripheral circulation in comparison with control lambs. The *in utero* antigenic exposure seemed to have increased the number of $CD5^+$ lymphocytes within the peripheral circulation. Pernthaner *et al.* (1996) have observed that susceptible lambs exhibited a higher proportion of $CD5^+$ lymphocytes in the peripheral circulation

than did resistant lambs before infection with *Trichostrongylus axei*. A similar observation was also made in the present study. Those *in utero* antigen-exposed lambs which had higher percentages of $CD5^+$ lymphocytes in the peripheral blood before challenge, had a correspondingly higher final worm count in the gut after challenge.

Three weeks after the live larval challenge there was a drop in the number of $CD5^+$ lymphocytes in the peripheral blood of *in utero* antigen-exposed lambs. In contrast, among the control lambs there was an increase in the $CD5^+$ lymphocyte levels at this time. It is possible that some of these circulating $CD5^+$ lymphocytes could have been modulated by the *in utero* antigenic exposure. On re-encounter with the homologous antigen in postnatal life these lymphocytes seems to be rapidly depleted from the peripheral circulation, possibly because of homing into the gut. Although there was no evidence of increased expression of $CD5^+$ in the jejunum the cells might be there as activated cells which no longer express CD5. Both the groups of lambs had significantly higher levels of $CD5^+$ lymphocytes in the peripheral blood eight weeks after challenge in comparison with the levels observed three weeks after challenge. The $CD5^+$ level eight weeks after challenge was marginally higher in the control group of lambs in comparison with the *in utero* antigen-exposed lambs.

ANTIGEN PRESENTING CELLS

INTRODUCTION

The cluster of differentiation antigen CD1 encompasses a group of relatively non-polymorphic molecules with genomic organisation similar to MHC I. CD1 consists of an α chain of molecular weight of 43-49 KDa, non-covalently associated with β2 microglobulin (Rhind *et al.*, 1996). Five genes have been described (CD1 a-e) and four protein products have been detected in humans. The differences in the molecular weight of the heavy chain between the various forms of CD1 molecules reflect differences in the extent of glycosylation (Hopkins & Dutia, 1991).

Studies have demonstrated the presence of at least two distinct subgroups of the ovine CD1 molecule. The monoclonal antibody SBU-T6 (20-27) recognises one such subgroup on the majority of circulating B cells, monocytes and tissue macrophages in addition to cortical thymocytes and dendritic cells (Mackay *et al.*, 1985; Rhind *et al.*, 1996). The cellular reactivity of cells identified by SBU-T6 suggests that this monoclonal antibody identifies the sheep homologue of CD1c. Owing to the wider cross reactivity of this monoclonal antibody it has also been regarded as a pan CD1 marker (Dutia & Hopkins, 1991; Hopkins & Dutia, 1991).

The monoclonal antibody CC20 recognises the ovine analogue of the human CD1b molecule. This antibody reacts only with thymocytes and dendritic cells (Dutia & Hopkins, 1991). Thus, it is much more specific than SBU-T6 in staining dendritic cells within tissues.

Dendritic cells (DCs) have been clearly shown to be the most competent "professional" antigen-presenting cells. DCs develop from bone marrow precursors. A small $CD34^+$ subset of haemopoietic progenitors gives rise to all blood cells and DCs (Banchereau & Steinman, 1998; Iwasaki & Kelsall, 1999). It is likely that the progeny of these cells colonise most tissues as immature non-dividing cells. DCs are present in most tissues in an 'immature state', unable to stimulate T cells. Although these DCs lack the requisite accessory signal for T cell activation, they are extremely well equipped to capture antigens and to participate in the induction of immunity. Captured antigens can induce full maturation and mobilisation of DCs (Banchereau & Steinman, 1998). Immature

DCs are able to capture antigens through macropinocytosis and receptor-mediated antigen uptake. They are extremely efficient in doing this as concentrations of antigens in the picomolar and nanomolar levels suffice to initiate an immune response (Banchereau & Steinman, 1998). However, once the DC has captured an antigen its further antigen uptake capability declines rapidly. DCs undergo maturation by assembling 'antigen MHC II complexes' on the cell surface where they remain stable for days (Banchereau & Steinman, 1998). To generate cytotoxic killer cells, which have the capacity to eliminate target cells, tumor and transplant, DCs have to present CD8-expressing T cells with antigenic peptides complexed to MHC I molecules. This is relatively straightforward if the DC is infected as in the case of a virus infection. It is less clear, however, how DCs would process and present antigen that has not had access to the cytosol in an MHC I restricted manner, for instance antigen derived from parasites and tumors. In such instances it is believed that DCs process dying cells which might be able to 'cross prime' or 'cross tolerise' T cells (Banchereau & Steinman, 1998).

Terminally differentiated or mature DCs can rapidly prime T cells. Once activated by DC, these T cells can complete the immune response by interacting with other cells, such as B cells for antibody formation, macrophages for cytokine release and targets for lysis. Thus, the two functions of dendritic cells are segregated in time: they first handle antigens and then, as mature DCs a day or more later, stimulate T cells.

A number of immunological studies have revealed the presence of DCs in various mucosa-associated lymphoid tissues. In the gut-associated lymphoid tissues, DCs are found in the Peyers's patches, lamina propria and draining mesenteric lymph nodes. In mature and foetal sheep, a high density of MHC II positive cells with dendritic morphology can be found in the lamina propria of the forestomach suggesting that the DCs present in the forestomach mucosa have a more constitutive character and are less dependent on antigenic stimulation (Josefsen & Landsverk, 1996). This observation raises the possibility that DCs present at different locations in the gut may have different functional capabilities. In contrast to T cells, the DCs do not vary between the different anatomical sites in the rumen.

DCs are present in the lamina propria throughout the small and large intestine of the mouse and human (Iwasaki & Kelsall, 1999). However, detailed functional studies of DCs from the lamina propria are lacking. In human colon, DCs form a reticular framework throughout the lamina propria and beneath the basement of the colonic crypts (Pavli *et al.*, 1995). In *Trichostrongylus*-infected sheep the lamina propria of the small intestine has an increased population of $CD1^+$ DCs. This increase in DCs is observed between 5-10 days post infection when the second phase of parasite rejection from the gastrointestinal tract is thought to occur (Emery *et al.*, 1993).

In the distal jejunal lymph node of calves the deep cortex contains a larger number of $CD1^+$ cells in comparison with adult cattle (Gunnes *et al.*, 1998). Gunnes *et al.* (1998) have argued that this could be the result of confrontation of calves with exogenous antigens in early postnatal life. In the mesenteric lymph nodes of cattle and sheep $CD1^+$ cells with a dendritic morphology are localized to the paracortical region (Hopkins & Dutia, 1991).

In foetal sheep between 68 and 135 days of gestation DCs can be demonstrated in the ileal Peyer's patches (Press *et al.*, 1992). MacKay *et al.* (1985) have demonstrated that less than 1 % of cells from the ileal Peyer's patches of adult sheep could be stained with the pan $CD1^+$ marker (SBU-T6).

Immunohistochemical analysis of Peyer's patches in the mouse has revealed two distinct populations of DCs. One subset of DCs is located immediately underneath the follicle-associated epithelium in the subepithelial dome. The DCs in the subepithelial dome are ideally situated anatomically for taking up luminal antigens transported by M cells (Kelsall & Strober, 1996). Similar MHC II- expressing cells with dendritic morphology have been found in the Peyer's patch subepithelial dome region of both humans and rats (Iwasaki & Kelsall, 1999). The other subset of DCs is present in the interfollicular region in close association with T cells. These DCs express antigens that correlate with maturation *in vitro*. This indirectly suggests that the interfollicular DCs are more mature than those in the subepithelial dome. The interfollicular DCs are most likely to be responsible for priming T cells, since they come in close contact with naive T cells.

Table- 6.1: Total positive area – Antigen presenting cells
MAb: SBU-T6

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	1302 ± 155	1039 ± 158	NS
Mesenteric lymph node	926 ± 161	1550 ± 248	P=.023
Ileal Peyer's patch	674 ± 149	601 ± 90	NS

Table- 6.2: Total positive area – Antigen presenting cells
MAb: CC20

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	188 ± 24	226 ± 52	NS
Mesenteric lymph node	688 ± 131	506 ± 66	NS
Ileal Peyer's patch	577 ± 70	814 ± 121	P=.05

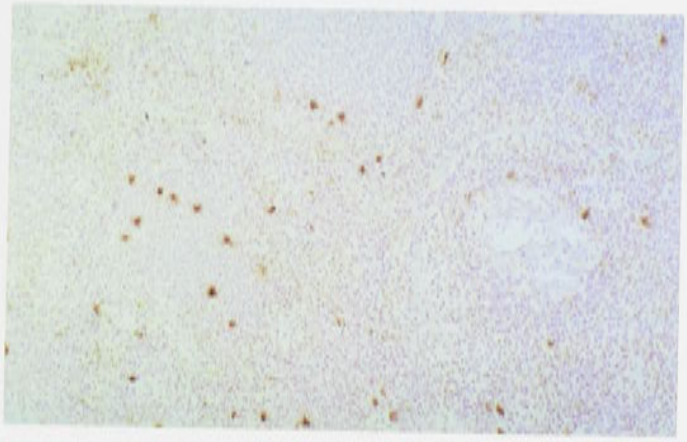
Table- 6.3: Ratio of SBU-T6 : CC20 –Total positive area stained

Tissue	Mean ± S.E.		Sig.
	In utero antigen-exposed	Control	
Jejunum	11.4 ± 3.4	10.9 ± 4.9	NS
Mesenteric lymph node	2.1 ± 0.4	4.7 ± 1.1	P=.015
Ileal Peyer's patch	1.2 ± 0.2	0.93 ± 0.2	NS

Fig-E: SBU-T6^{+} dendritic cells within the MLN of *in utero* antigen-exposed lambs. The area staining for SBU-T6 was significantly lesser within the MLN of *in utero* antigen-exposed lambs in comparison with control lambs as assessed by image analysis.

Fig-F: SBU-T6^{+} dendritic cells within the MLN of control lambs. The area staining for SBU-T6 was significantly greater within the MLN of control lambs in comparison with *in utero* antigen-exposed as assessed by image analysis.

Fig-E:



40 μm

Fig-F:

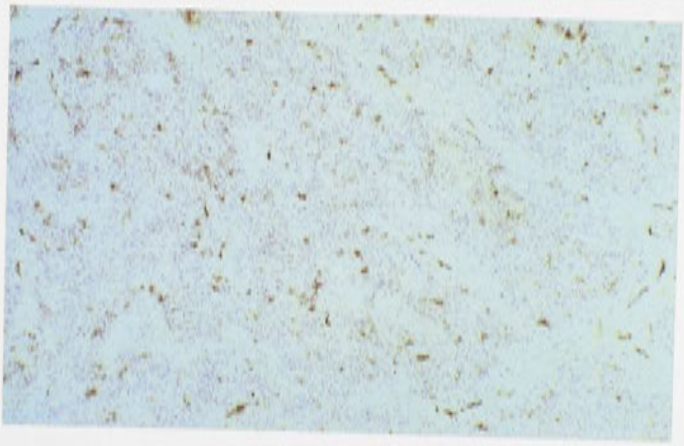


Fig-G: SBU-T6 positive cells within the IPP. Positive cells are seen distributed in the mucosa of the IPP, with many of them clustered towards the luminal end of the villi close to the mucosal epithelium.

Fig-G:

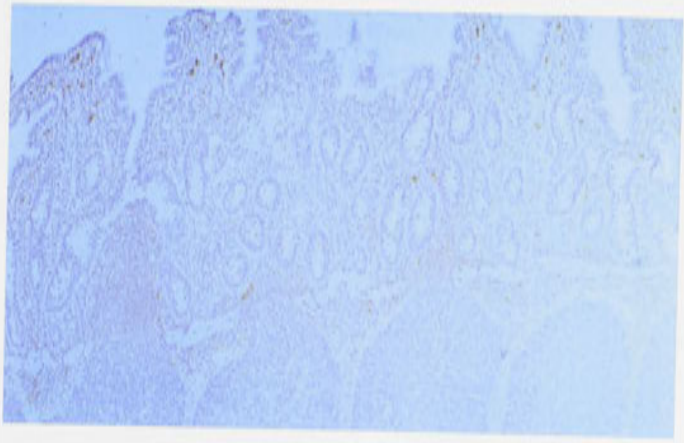
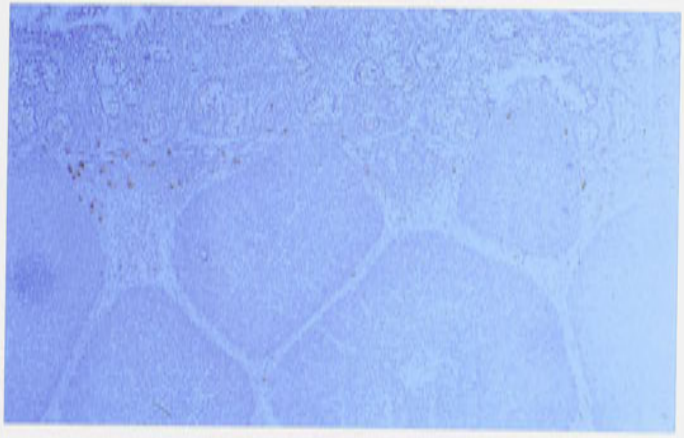


Fig-H: $CC20^{+}$ dendritic cells distributed among the lymphocytes of the interfollicular region of the IPP of *in utero* antigen-exposed lambs. The area staining for CC20 was significantly lesser in the *in utero* antigen-exposed group in comparison with control lambs as assessed by image analysis.

Fig-I: $CC20^{+}$ dendritic cells distributed among the lymphocytes of the interfollicular region of the IPP of control lambs. The area staining for CC20 was significantly greater in the control group in comparison with *in utero* antigen-exposed lambs as assessed by image analysis.

Fig-H:



40 μm

Fig-I:

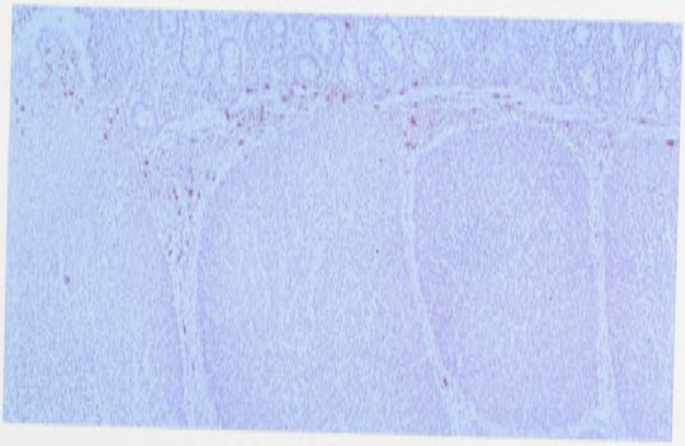
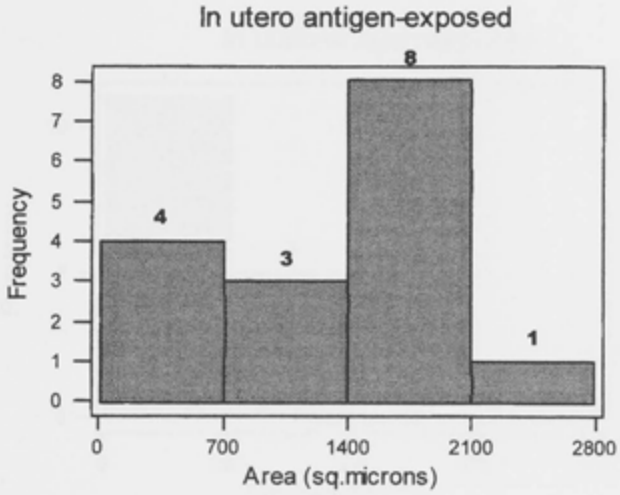


Fig-6.4: Frequency of occurrence of APC in Jejunum stained with MAb SBU-T6



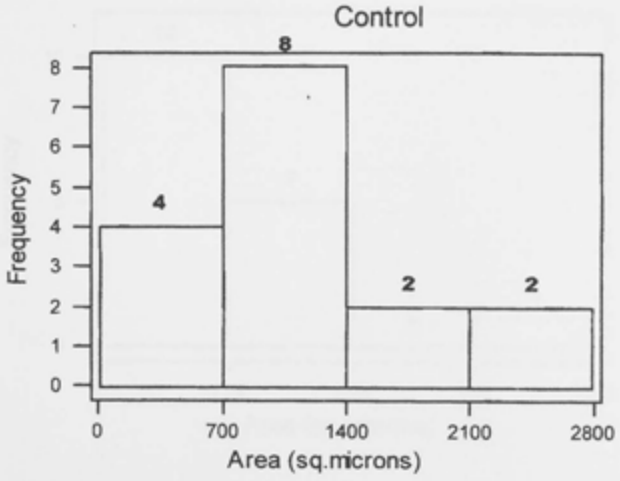
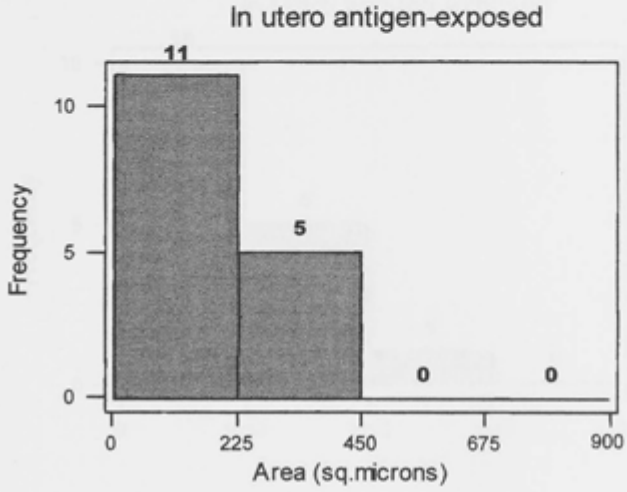


Fig-6.5: Frequency of occurrence of APC in Jejunum stained with MAb CC20



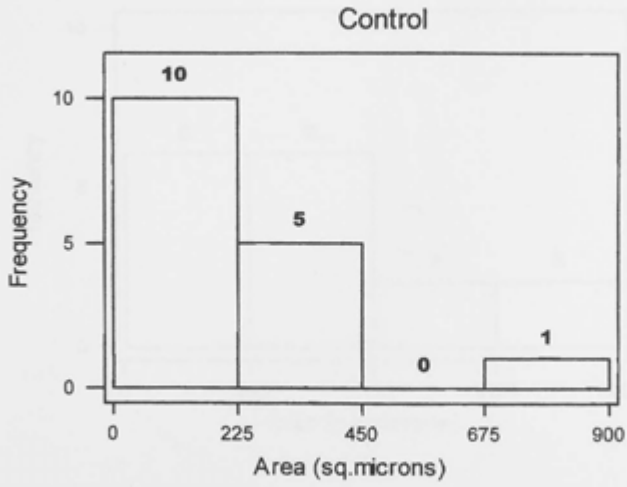
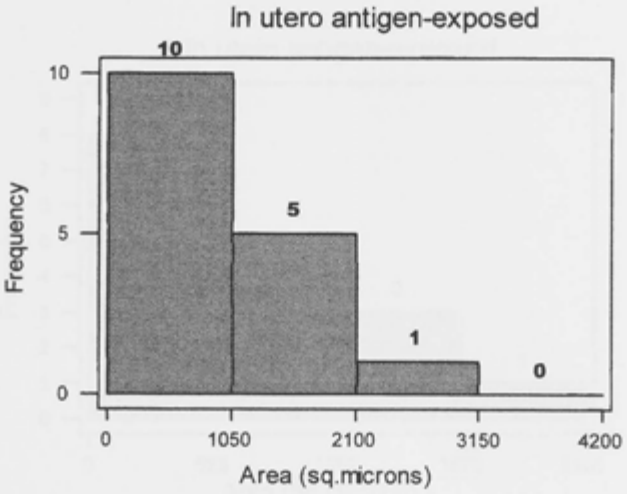


Fig-6.6 : Frequency of occurrence of APC in MLN stained with MAb SBU-T6



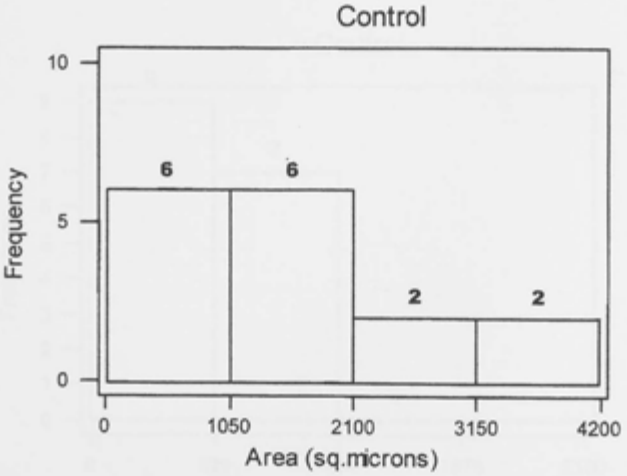
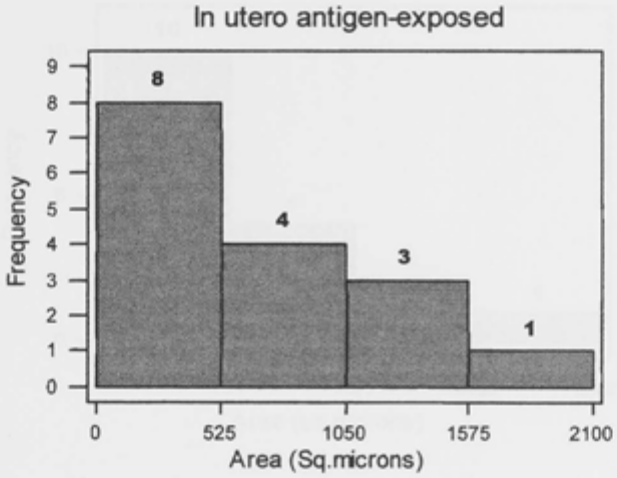


Fig-6.7: Frequency of occurrence of APC in MLN stained with MAb CC20



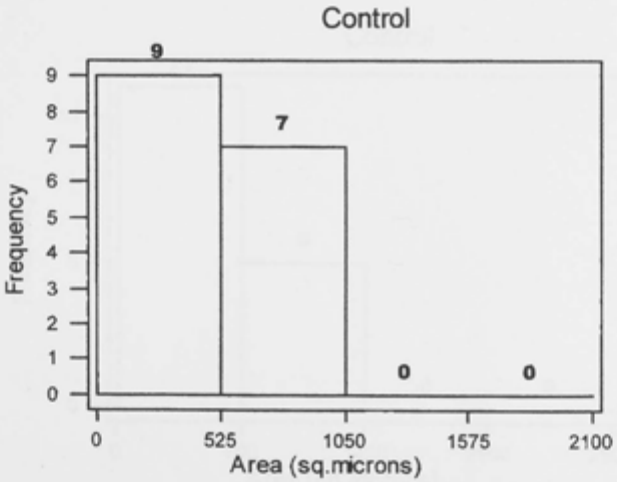
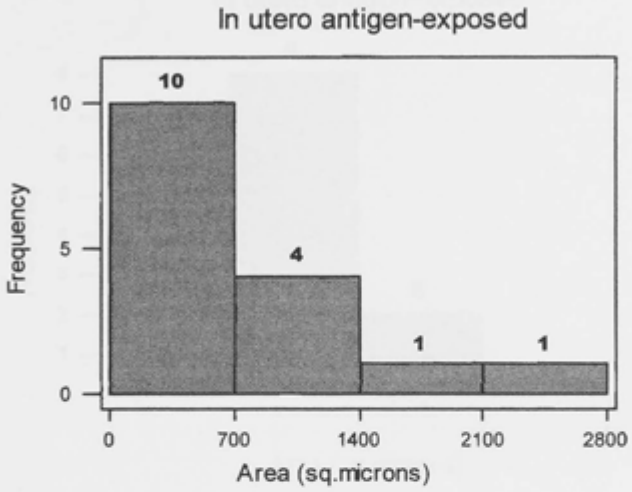


Fig-6.8 : Frequency of occurrence of APC in IPP stained with MAb SBU- T6



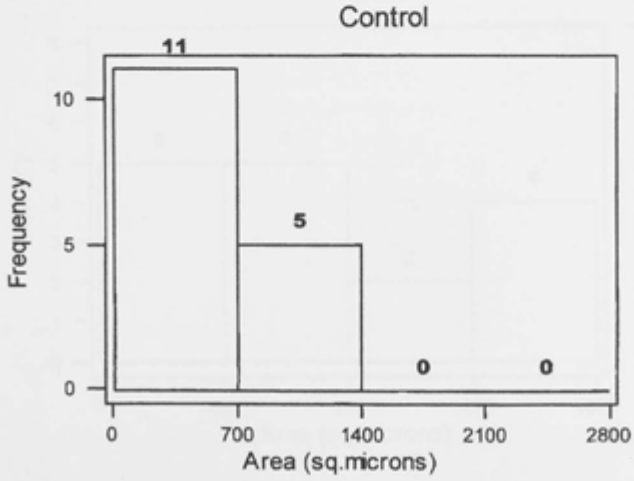
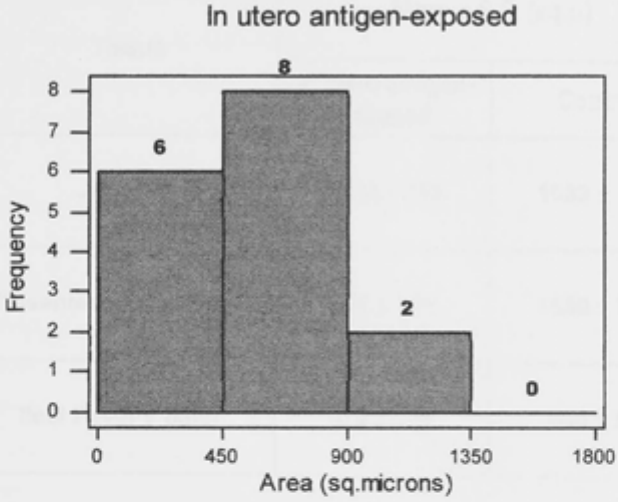


Fig-6.9: Frequency of occurrence of APC in IPP stained with MAb CC20



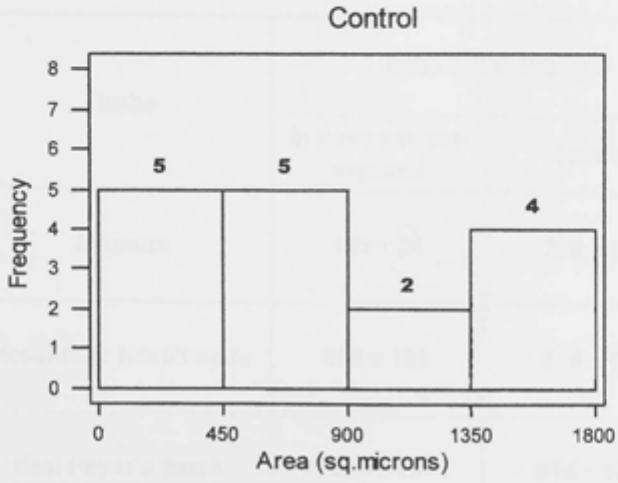


Table- 6.1: Total positive area – Antigen presenting cells
MAb: SBU-T6

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	1302 ± 155	1039 ± 158	NS
Mesenteric lymph node	926 ± 161	1550 ± 248	P=.023
Ileal Peyer's patch	674 ± 149	601 ± 90	NS

Table- 6.2: Total positive area – Antigen presenting cells
MAb: CC20

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	188 ± 24	226 ± 52	NS
Mesenteric lymph node	688 ± 131	506 ± 66	NS
Ileal Peyer's patch	577 ± 70	814 ± 121	P=.05

The presence of distinct DCs in the mucosal immune system may be especially conducive to the differentiation of T cells into Th2/Th3 pathways. DCs are capable of producing IL-10 and TGF-β when they interact with naïve T cells. These cytokines either directly or indirectly drive T cells within the gut-associated lymphoid tissue to differentiate into T cells producing Th2 cytokines and TGF-β (Iwasaki & Kelsall, 1999).

The following hypothesis has been put forward to explain the role played by DCs in the induction of the response of T cells in the gut (Iwasaki & Kelsall, 1999). Subepithelial dome DCs take up intestinal antigens transported via M cells and migrate to the T cell regions and become interfollicular DCs. During migration, subepithelial DCs can enter either of two distinct development pathways. If the antigen encountered is a non-infectious food antigen the default pathway for the interfollicular DCs is to generate Th2 and/or Th3 responses through secretion of high levels of IL-10 and TGF-β and low levels of IL-12. However, when encounters with microbial stimuli, such as double stranded RNA or lipopolysaccharides occur, conventional maturation of DCs is triggered. This maturation of DCs leads to the secretion of high levels of IL-12, which induces T cells to secrete interferon γ resulting in a Th1 response.

RESULTS

Jejunum:

Staining with Monoclonal antibody SBU-T6:

Histology: It was noted that the monoclonal antibody SBU-T6 stained cells which were morphologically distinctly different from lymphocytes . These stained cells were much larger than lymphocytes and under high power (40x), many were observed to have small cytoplasmic projections protruding from their cell membranes. The intensity of staining with this monoclonal antibody ranged from medium to high. In the jejunum, the intensity of staining with monoclonal antibody SBU-T6 tended to be slightly greater than that of staining with monoclonal antibody CC20. The stained cells were usually found in small clusters (2-5 cells) or randomly distributed. Most of the positive cells were localized within the villi of the mucosa. Some of the cells were found aggregated at the extreme luminal end of the villi in association with the epithelium. There

were also a few positive cells distributed within the lamina propria. This was in contrast to the staining with monoclonal antibody CC20 after which most of the positive cells were localized to the lamina propria. No other histological differences between the two groups were apparent.

Image analysis: The total area stained with the monoclonal antibody SBU-T6 was not significantly different between the two groups of animals (Table-6.1). However, there was a tendency for the mean stained area to be marginally more extensive (20.2 %) in the *in utero* antigen-exposed group in comparison with the control group.

If the area stained by the monoclonal antibody SBU-T6 in the jejunum was divided into four equal ranges and correlated with the frequency distribution of animals, distinct differences between the two groups became evident. In the *in utero* antigen-exposed group (Fig-6.4) 50 % of the animals had a positive area within the range of >1400 and <2100 sq.μ. In the control group (Fig-6.4) only two of animals fell within this range (>1400 and <2100 sq.μ). In the control group the majority of animals (8/16) had a positive area that fell within the range >700 and <1400 sq.μ while there were only three animals in the *in utero* antigen-exposed group within the same range.

Staining with monoclonal antibody CC20 :

Histology: The cells stained were larger than lymphocytes. Examination under high power (40X) revealed that, unlike the smoother cell membranes of lymphocytes some of the positive cells had structures like dendrites protruding from the cell membranes. The intensity of staining varied from low to medium. The vast majority of the positive cells tended to be localized in the lamina propria and to be distributed either in small clusters (2-4 cells) or randomly. In some instances the positive cells were even seen very close to the muscularis mucosa. Occasionally, positive cells were seen randomly distributed within the villi of the mucosa. There were no histological differences observed between the control and *in utero* antigen-exposed animals.

Image analysis: The total area stained by the monoclonal antibody CC20 in the jejunum did not differ significantly between the two group of animals (Table-6.2). There was a marginal tendency for the mean level to be higher (16.8 %) in the control group in comparison to the *in utero* antigen-exposed lambs.

When the area stained by the monoclonal antibody in the jejunum was divided into four equal ranges and correlated with the frequency distribution of animals very little difference was apparent between the two groups (Fig-6.5).

Ratio of total positive area of SBU-T6 and CC20 staining cells: The ratio was not significantly different between the two groups of lambs (Table-6.3).

Mesenteric lymph node:

Staining with monoclonal antibody SBU-T6:

Histology: Positive cells had morphological features distinct from the surrounding lymphocytes. The cells were much larger with irregular borders in comparison with lymphocytes as seen under high power (40X). They were usually stained with light to medium intensity. Many of these cells were seen in patchy clusters between the trabeculae in close association with lymphocytes. In the MLN, the intensity of staining with the monoclonal antibody SBU-T6 was almost identical with that of monoclonal antibody CC20. However the SBU-T6 antibody seemed to stain some cells which were distinctly different from those stained by CC20.

Image analysis: In the MLN, the total area stained with the monoclonal antibody SBU-T6 was very high in the control group of animals. The mean level was significantly higher (P=.023) in the control group of animals in comparison with the *in utero* antigen-exposed group (Table-6.1, Fig-E & F).

When the area stained by the SBU-T6 antibody in the MLN was divided into four equal ranges and the frequency distribution of animals plotted, distinct differences became evident between the two groups. In the *in utero* antigen-exposed group (Fig-6.6) there was only one animal with a positive area >2100 sq.μ. However, in the control group (Fig-6.6) there were four animals (25 %) with an area >2100 sq.μ. In the *in utero* antigen-exposed group the majority of animals (10/16) had a small area (>0 and <1050 sq.μ) staining with the monoclonal antibody SBU-T6 whereas in the control group there were only 6/16 animals with a small stained area (>0 and <1050 sq.μ)

Staining with monoclonal antibody CC20 :

Histology: The positive cells were much larger that the surrounding lymphocytes. Some of the cells had dendritic morphology. The intensity of staining was usually moderate. However, on rare occasions, in some animals, the

cells were more darkly stained. In general, most of the positive cells stained with the monoclonal antibody CC20 tended to be more lightly stained than those cells stained with the monoclonal antibody SBU-T6. The positive cells were usually aggregated in small clusters (2-5 cells) between the trabeculae. However, randomly distributed cells were also commonly seen in many sections. No histological differences could be observed between the two groups.

Image analysis: The area stained by the monoclonal antibody CC20 in the MLN was not statistically different between the two groups (Table-6.2). However, it was observed that the mean positive area was greater by 26.5 % in the *in utero* antigen-exposed group in comparison with that of the control group.

When the area stained by the CC20 antibody was divided into four equal ranges and the frequency distribution of animals calculated, distinct differences between the two groups were evident. In the *in utero* antigen-exposed group (Fig-6.7) there were four animals (25 %) having a positive area >1050 sq.μ. In the control group there were no animals with an area >1050 sq.μ (Fig-6.7).

Ratio of total positive area of SBU-T6 and CC20 staining cells: The ratio was significantly higher ($p=.015$) in the control group of animals in comparison with the *in utero* antigen-exposed lambs (Table-6.3).

Ileal Peyer's patch:

Staining with monoclonal antibody SBU-T6 :

Histology: Positive cells were larger and had a dendritic morphology distinct from lymphocytes. They tended to be stained with low to medium intensity. The SBU-T6 antibody tended to stain with slightly lower intensity than the monoclonal antibody CC20 in the IPP. Unlike $CC20^+$ cells, most of the cells stained with the SBU-T6 antibody tended to be distributed in the mucosa of the ileum (Fig-G). Many of them were clustered in small patches towards the luminal end of the villi, sometimes in the subepithelial dome region. A few positive cells were also found randomly distributed in the interfollicular region and in close association with the connective tissue capsule of the follicle.

Image analysis: The total area stained by the monoclonal antibody SBU-T6 in the two groups of animals (Table-6.1) was not statistically different. There was a marginal tendency for the mean ± SE levels to be higher in the *in utero* antigen-exposed group of animals in comparison with the control group.

When the area occupied by the positive cells in the IPP was divided into four equal ranges and correlated with the frequency distribution of animals, very little difference was evident between the two groups (Fig-6.8). There was a marginal tendency for the stained area (>1400 sq.μ) to be greater in the *in utero* antigen-exposed group with 2/16 animals being in this category compared with none in the control group.

Staining with monoclonal antibody CC20 :

Histology: Positive cells were morphologically larger than the surrounding lymphocytes. The positive cells were usually darker staining than the ones observed within the jejunum and MLN. The vast majority of cells were found in close association with the lymphocytes of the interfollicular region. They were usually distributed randomly or in small clusters between the lymphocytes of the interfollicular region (Fig-H & I). There were also a few positive cells distributed within the corona and the dome regions. Likewise, positive cells could sometimes be seen close to the connective tissue capsule surrounding the follicle. Rarely, $CC20^+$ positive cells were also seen infiltrating into the medullary region of the follicles.

Image analysis: The mean area stained by the Monoclonal antibody CC20 was significantly greater (P=.05) in the control group of animals compared with the *in utero* antigen-exposed group (Table-6.2, Fig-H & I).

When the area occupied by the positive cells in the IPP was divided into four equal ranges and correlated with the frequency distribution of animals, clear cut differences between the two groups became evident. In the *in utero* antigen-exposed group there were no animals (Fig-6.9) with an area >1350 sq.μ. On the other hand in the control group (Fig-6.9) there were four animals (25 %) with a positive area >1350 sq.μ.

Ratio of total positive area of SBU-T6 and CC20 staining cells: The ratio was not significantly different between the two groups of lambs (Table-6.3).

FACS analysis of peripheral blood with monoclonal antibody CC20: This analysis revealed marked changes in the dynamics of $CC20^+$ cells at various points of time (Table-6.10 and Fig-6.11). Before challenge with the live larvae, the level of $CC20^+$ cells tended to be higher in the peripheral circulation of *in utero* antigen-exposed lambs in comparison with control lambs (Table-6.10).

Three weeks after challenge, there was a significant increase (p=.031) in the number of $CC20^+$ cells among control lambs, in comparison with the pre-challenge level (Table-6.10 and Fig-6.11). On the contrary, among *in utero* antigen-exposed lambs there was a marked drop (not significant statistically) in the number of positive cells three weeks after challenge in comparison with pre-challenge levels. Both of these features contributed to the significantly different levels of $CC20^+$ cells detected in the peripheral circulation of control and *in utero* antigen-exposed lambs at three weeks post-challenge. The mean levels three weeks after challenge were significantly greater (p=.036) in control lambs in comparison with *in utero* antigen-exposed lambs (Table-6.10). Among control lambs the high levels of positive cells detected three weeks after challenge again dropped to significantly (p=.036) low levels by eight weeks.

DISCUSSION

The CD1 molecules expressed on the surface of professional antigen-presenting cells are structurally homologous to the major histocompatibility complex class I and class II molecules. Two monoclonal antibodies SBU-T6 and CC20 were used for the immunohistochemical characterisation of DCs in the gut tissue. It has been proposed that the monoclonal antibody SBU-T6 with its wider cross reactivity is a pan CD1 marker that identifies the sheep homologue of CD1c (Dutia & Hopkins, 1991; Hopkins & Dutia, 1991). The monoclonal antibody CC20 has a much more limited cross reactivity to tissues than SBU-T6 and recognises the CD1b molecule (Dutia & Hopkins, 1991; Howard *et al.*, 1993). Although the difference was not statistically significant, the levels of $CC20^+$ cells in the peripheral circulation of unchallenged, *in utero* antigen-exposed lambs were four times higher than in control lambs. The levels of $CC20^+$ cells detected in the peripheral circulation of lambs prior to challenge were well within the range of 1-2 % reported in literature (Hopkins & Dutia, 1991). However, challenge with the live larvae brought about distinctly different changes in the peripheral levels of $CC20^+$ cells which suggested that this subpopulation had been modified by previous antigenic exposure in those lambs orally exposed to antigen *in utero*. Within three weeks of challenge, there was a significant increase in the number of $CC20^+$ cells in the peripheral circulation of control animals. This change in the peripheral blood possibly reflects detection of $CC20^+$ cells that are

in the phase of active migration to the gut as the target site of infection. On the contrary, three weeks after challenge of *in utero* antigen-exposed lambs there was a 65 % drop (not statistically significant) in the number of $CC20^+$ cells in the peripheral circulation. This suggests that a majority of $CC20^+$ cells may have been depleted from the peripheral circulation and retained in the infected gut. Among *in utero* antigen-exposed lambs, many of the $CC20^+$ cells in the peripheral circulation could have been pre-sensitised with the larval antigen *in utero*. On re-exposure to the homologous antigens in post-natal life they appear to have recognised the challenge antigen and responded in an anamnestic manner. The CC20 levels that increased in the peripheral circulation of control lambs at three weeks post-challenge again dropped to significantly low levels by eight weeks. This may indicate that cells of the control lambs took a longer time to home into the gut a typical feature of a primary infection.

The cells stained by both these monoclonal antibodies in the various gut tissues had a dendritic morphology identical to that reported in the literature (Hopkins & Dutia, 1991; Mackay *et al*., 1985; Rhind *et al*., 1996). The two monoclonal antibodies however seem to stain different populations of DCs within the various tissues.

In the jejunum and IPP, the distribution of $SBU\text{-}T6^+$ DCs was similar. These $SBU\text{-}T6^+$ cells were often clustered towards the luminal end of the villi and in the subepithelial dome region of the IPP. On the contrary, most CC20-staining DCs were found deep in the jejunal lamina propria and in close association with the T lymphocytes of the IPP interfollicular region. This is likely to reflect the presence of two discrete cell populations rather than indicating that one is a subset of the other. Immunohistochemical analysis of the Peyer's patches in the mouse has also revealed two distinct populations of DCs. One subset of murine DCs is localized immediately underneath the follicle associated epithelium in the subepithelial dome (Kelsall & Strober, 1996). Similar MHC class II-expressing cells with dendritic morphology have been found in the subepithelial dome region of the Peyer's patches of both humans and rats (Iwasaki & Kelsall, 1999). The DCs in the subepithelial dome are ideally suited anatomically for taking up luminal antigens transported from the intestinal lumen by the M cells (Iwasaki & Kelsall, 1999; Kelsall & Strober, 1996). The subepithelial dome DCs of mice

have considerable similarity to the cells stained by the monoclonal antibody SBU-T6 in the IPP of lambs in the present experiments. The mucosal distribution of SBU-T6$^+$ DCs in the jejunum and the IPP strongly suggests that they are poised for the capture and presentation of gut lumen antigens to T cells.

The second subset of DCs in the mouse Peyer's patch is present in the interfollicular region in close association with T cells. These interfollicular DCs are considered to be responsible for priming T cells since they are in close contact with naïve T cells (Iwasaki & Kelsall, 1999; Kelsall & Strober, 1996). In mice, there are suggestions that these interfollicular DCs might be more mature or differentiated, an observation based on the expression of certain maturation specific markers (Iwasaki & Kelsall, 1999). In humans, the *in vitro* expression of CD1b is correlated with the activation of DCs (Emile *et al.*, 1994). In the present study, the majority of CD1b$^+$ DCs, which stained with the monoclonal antibody CC20, were found in the interfollicular region in close association with T lymphocytes. It appears that, in lambs, CC20$^+$ DCs may be differentiated cells and may play a vital role in the induction of T cells in the interfollicular region of IPP and in the lamina propria of the jejunum.

Rhind *et al.* (1996) have reported that, in sheep, cells stained by the monoclonal antibody SBU-T6 are much more widely distributed than those stained with the CC20 antibody. In the present study this was true in the case of the jejunum which had extremely high levels of SBU-T6 stained cells in comparison with those stained by CC20. The average SBU-T6:CC20 ratio was around 11:1 in both groups of animals. The MLN also had considerably higher levels of SBU-T6 staining DCs in comparison with CC20$^+$ DCs. The ratio ranged from 2 to 5:1 among both groups of animals. This high ratio may reflect higher rates of immigration, local proliferation or retention of SBU-T6$^+$ DC, or a higher rate of emigration of CC20$^+$ DCs, and suggests that jejunum and MLN are active sites with respect to the function (possibly antigen capture) of SBU-T6$^+$ DC. This is consistent with the high antigenic load to which jejunum is exposed.

In contrast, DCs staining with the monoclonal antibodies SBU-T6 and CC20 were found in almost equal proportions in the IPP. The SBU-T6:CC20 ratio ranged from 0.9-1.2:1. Thus it appears that the observation of Rhind *et al.* (1996) that SBU-T6 predominates in tissues does not hold true in the IPP of

lambs. This might reflect a low level of antigen capture in this tissue, or a rapid rate of differentiation and maturation of SBU-T6$^+$ DCs into CC20$^+$ DCs.

In the jejunum of *in utero* antigen-exposed lambs there was a tendency for the levels of SBU-T6$^+$ DCs to be marginally higher (20 %) in comparison with control animals. A similar observation has also been made by McClure *et al.* (1992) who observed large number of CD1$^+$ within the villi of immune animals that were challenged with *T. colubriformis*. This study does not indicate whether the increased presence of SBU-T6$^+$ DCs in the jejunum of *in utero* antigen-exposed lambs resulted from increased immigration, proliferation or retention of these cells. It has been proposed that TNF-α is involved in the migration of DCs within the gut (MacPherson *et al.*, 1995). The increased levels of TNF-α in the jejunum of *in utero* antigen-exposed lambs could have been one reason favouring the increased migration of DCs into the jejunum. Thus it appears that, in the *in utero* antigen-exposed lambs, there were greater number of DCs available in the mucosa of the jejunum to capture antigens from the gut lumen. However, this increased number of SBU-T6$^+$ DCs within the jejunum of *in utero* antigen-exposed lambs was not effective in mounting an immune response that could clear the parasite from the gut. Rather, the marginally higher worm count in the gut of *in utero* antigen-exposed lambs suggested a tolerised immune response to the parasite even with the increased level of inflammatory response. Other studies have also demonstrated that expanding the DC population within the gut could actually enhance the induction of tolerance (Viney *et al.*, 1998).

In contrast with SBU-T6$^+$ DCs the number of CC20$^+$ DCs within the jejunum of *in utero* antigen-exposed lambs tended to be marginally lower (17 %) than in control lambs. It is possible that, in the *in utero* antigen-exposed lambs, these CC20$^+$ DCs are rapidly migrating to other regions of the gut like the MLN which had considerably larger amounts of these cells.

In the IPP the area stained by the mucosa associated DCs (SBU-T6$^+$) was only marginally higher (11 %) in the *in utero* antigen-exposed group of lambs. In direct contrast, CC20$^+$ DCs which occurred predominantly in the interfollicular region were found in significantly higher numbers in the IPP of control lambs compared with *in utero* antigen-exposed lambs. Thus *in utero* oral antigenic

exposure followed by re-exposure to the same antigen in post-natal life can significantly influence the levels of the CD1b expressing subpopulation of DCs within the IPP. It is hypothesised that these DCs seem to be still priming the T cells in the interfollicular region of control lambs. On the contrary, it appears that a substantial number of $CC20^+$ DCs have migrated from the IPP of *in utero* antigen-exposed lambs and homed into the MLN. This possibility was supported by the observed trend for MLN of *in utero* antigen-exposed lambs to have considerably higher (26.5 %) levels of $CC20^+$ DCs in comparison with control lambs. This increase in MLN $CC20^+$ DC was associated with increased amounts of the pro-inflammatory cytokine TNF-α.

The ratio of SBU-T6:CC20 staining DCs was significantly greater in the MLN of control lambs. It appears that the MLN of *Trichostrongylus*-infected lambs might reflect the dynamics of DC activation. The expression of CD1b on DCs is correlated with its activation/ differentiation. If this aspect is taken into account, the SBU-T6:CC20 staining ratio gives an indication that *in utero* antigenic exposure of the gut mucosal immune system followed by post natal exposure of the homologous antigens favours increased maturation and differentiation of $CD1b^+$ DCs in the MLN.

CD1b expressing DCs are thought to play a very important role in the development of interstitial inflammation (Cuzic *et al.*, 1992). Increased levels of $CD1b^+$ DCs have been observed in inflammatory conditions like glomerulonephritis (Cuzic *et al.*, 1992) and multiple sclerosis (Battistini *et al.*, 1996). *In vitro* studies have also shown that CD1b restricted T cell lines can behave similarly to classically-restricted Th1-type T cells. CD1b-restricted T cells may regulate immune responses to pathogens by simultaneously enhancing cell mediated immunity and down-regulating IgG4 and IgE responses (Fujieda *et al.*, 1998). In the context of the present study, although the memory and homing capabilities of $CD1b^+$ DCs from foetal lambs seem to be intact, they were not able to initiate a protective effector response in the gut as evidenced by failure to reduce numbers of parasites in the gut. Thus it appears that the $CD1b^+$ DC-initiated response may be inappropriate in this disease context.

GOBLET CELLS

INTRODUCTION

Goblet cells reside throughout the length of the small and large intestinal epithelium. They are highly polarised exocrine cells recognisable by their accumulation of secretory granules. Goblet cells arise by mitosis from multipotential stem cells at the base of the crypts (Cheng & Leblond, 1974). In the course of their differentiation, these stem cells migrate from the base of the crypts to the villous tip, from where they are sloughed into the lumen. This progression from cell birth to death occurs in mice over the course of 2-3 days; thus the population of goblet cells is very short lived and is constantly undergoing replacement (Specian & Oliver, 1991) Goblet cells synthesise and secrete high molecular weight glycoproteins called mucins. Upon secretion, mucins hydrate and gel, generating a protective mucus blanket overlying the epithelium (Specian & Oliver, 1991; Verdugo, 1990). The intestinal mucins are key components of the first line of defense against intestinal pathogens (Belley *et al.*, 1999). Goblet cells are also known to secrete important factors like terfolins. These peptides protect the gut epithelium and promote healing after injury (Ogata & Podolsky, 1997). In gastrointestinal parasitic infections, mucin is thought to play a protective role by trapping incoming parasites and preventing their establishment.

Many intestinal nematode infections induce a goblet cell hyperplasia and an increase in mucin production (Else & Finkelman, 1998). At least part of these changes is thought to be under the control of T lymphocytes. Among the earliest work suggesting this link was that of Garside *et al.* (1992). They observed that oral administration to mice of the larvae of *Trichinella spiralis* leads to an enteropathy characterised by villous atrophy, crypt hyperplasia and goblet cell hyperplasia. These changes could be prevented by the administration of cyclosporin A and hence they believed that the enteropathy that was induced was dependent on T helper cells. The importance of T cells in the induction of goblet cell hyperplasia was also demonstrated by Ishikawa *et al.* (1993) who observed that, in hypothymic rats infected with *Nippostrongylus brasiliensis*, there was no increase in intestinal goblet cell numbers.

Ishikawa *et al.* (1997) carried out a detailed analysis of the role of T cells in the regulation of small intestinal goblet cells in mice infected with the parasite *Trichinella spiralis.* They observed that small intestinal goblet cell hyperplasia occurred eight days after infection when expulsion of worms began from the gut. During this period, lymphocytes in the draining mesenteric lymph node underwent a predominant Th2 type response as shown by enhanced *in vitro* production of IL-5 but not of interferon γ. Ishikawa *et al.* (1997) were also able to demonstrate that the adoptive transfer of Th2-enriched cells further enhanced goblet cell hyperplasia in infected, recipient mice.

Studies have indicated that the physiochemical nature of the mucin produced by the goblet cells is critical for the expulsion of parasites from the gut. Shi *et al.* (1994) evaluated the goblet cell response in the jejunum of four genera of hamsters after infection with *Strongyloides venezuelensis*. In all four genera, goblet cell hyperplasia was observed at the time of expulsion of the parasite. However, in the comparative study of the four genera the degree of goblet cell hyperplasia did not correlate with the rapidity of expulsion of the parasite. On the other hand, the rapidity of expulsion of *S. venezuelensis* closely correlated with the degree of sulphation of goblet cell mucin. Two among the four genera of hamsters which had highly sulphated goblet cell mucins showed the fastest expulsion of the parasite in comparison with the other two genera which had less sulphated mucins.

There is also a suggestion that the goblet cell response of susceptible and resistant strains of animals to a primary parasitic infection might differ both qualitatively and quantitatively. Manjili *et al.* (1998) compared small intestine goblet cell numbers and mucus composition in guinea pigs with genetically determined differences in responsiveness to *Trichostrongylus colubriformis* infection. Prior to infection, no differences were detected between the susceptible and resistant animals. However, following primary infection with the parasite, pronounced goblet cell hyperplasia developed and the proportion of sulphomucin in these animals increased. Both these changes developed significantly earlier in the resistant strain of guinea pigs.

Goblet cell responses to gastrointestinal nematode infections have also been reported in sheep, although to a limited extent. A common feature of lambs

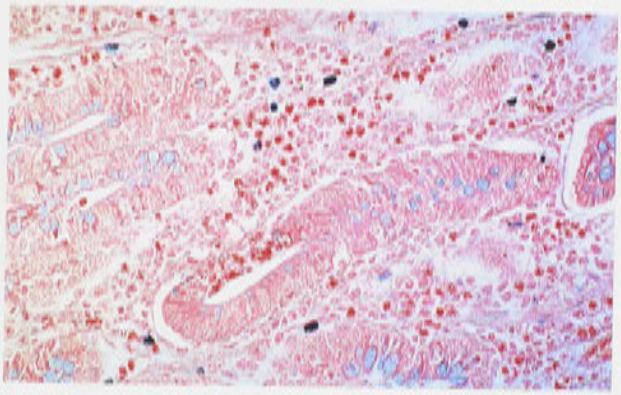
Table- 7.1: Goblet cell numbers

Tissue	Mean ± S.E. (numbers)		Sig.
	In utero antigen-exposed	Control	
Jejunum	99.1 ± 11	124.2 ± 9.2	.043

Fig-J: Goblet cells distributed within the jejunum of *in utero* antigen-exposed lambs. The goblet cell numbers were significantly lesser in the *in utero* antigen-exposed group in comparison with control lambs.

Fig-K: Goblet cells distributed within the jejunum of control lambs. The goblet cell numbers were significantly greater in the control group in comparison with *in utero* antigen-exposed lambs. Cells tended to be darker staining and had larger cell volume in comparison with *in utero* antigen-exposed lambs.

Fig-J:



80 μm

Fig-K:

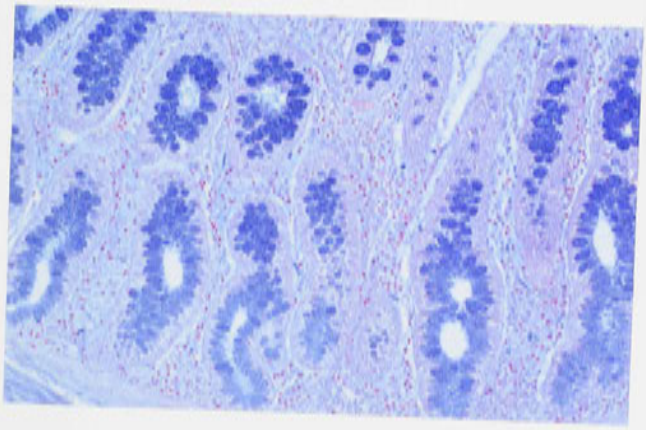
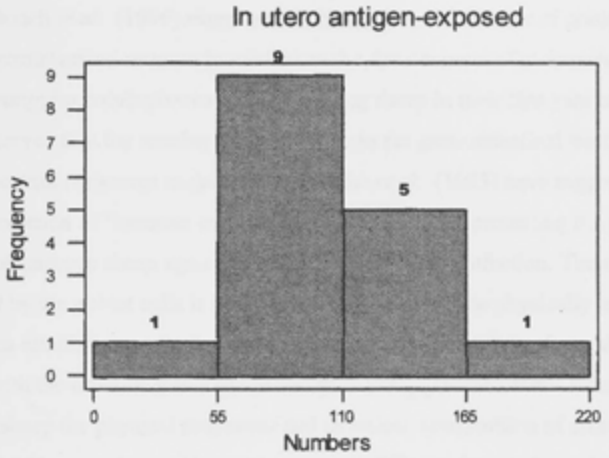
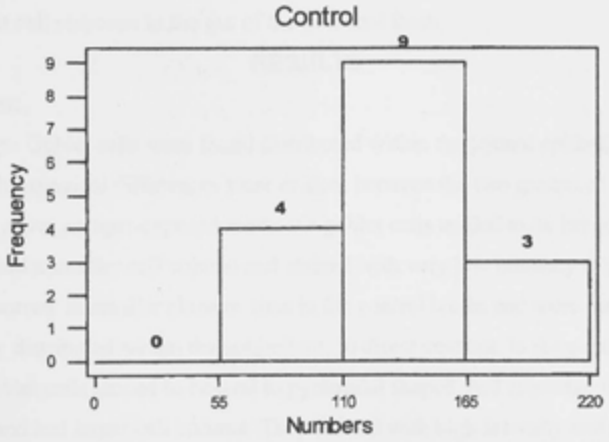


Fig-7.2: Frequency of occurrence of goblet cell in jejunum





chronically infected with *Trichostrongylus colubriformis* is goblet cell hyperplasia. The goblet cell hyperplasia was always observed irrespective of the parasitic burden in the gut (Angus & Coop, 1984).

Douch *et al.* (1986) examined the changes in the number of goblet cells in the gastrointestinal mucosa in relation to the development of resistance to *Trichostrongylus colubriformis* among grazing sheep in their first year of life. They observed that the number of goblet cells in the gastrointestinal tract was not correlated with resistance to the parasite. Miller *et al.* (1983) have suggested that the phenomenon of 'immune exclusion' is responsible for protecting the gut mucosa of immune sheep against *Haemonchus contortus* infection. The mucus produced by the goblet cells is thought to entrap the parasite physically and thus prevent its establishment in the gut. The quality of the mucus has been shown to change with the immune status of the sheep. Among gastrointestinal nematode resistant sheep the physical properties and chemical composition of muco-polysaccharide, proteins and leucotriene are all different from naïve animals (Douch *et al.*, 1983; Jones *et al.*, 1994). It would be interesting to find out if interfering with the developing gut immune system of the foetal lamb can alter the goblet cell response in the gut of the perinatal lamb.

RESULTS

Jejunum:

Histology: Goblet cells were found distributed within the jejunal epithelium. Distinct histological differences were evident between the two groups of lambs. In the *in utero* antigen-exposed lambs the goblet cells tended to be irregularly shaped, had a smaller cell volume and stained with very low intensity (Fig-J). They occurred in smaller clusters than in the control lambs and were sometimes randomly distributed within the epithelium. In direct contrast, in the control lambs goblet cells tended to be oval to pyramidal shaped, had smoother cell borders and had larger cell volume. They stained with high intensity and tended to be clustered in large numbers within the epithelium (Fig-K).

Cell count: The number of goblet cells per microscopic field (40X) was significantly greater (.043) in the control group of lambs in comparison with the *in utero* antigen-exposed lambs (Table-7.1).

When the goblet cell distribution in the jejunum was divided into four equal ranges distinct differences were observed between the frequency of animals in each range. In the *in utero* antigen-exposed lambs (Fig-7.2) the majority of animals (10/16) had goblet cell numbers <110. Among control lambs (Fig-7.2) there were only 4/16 animals with such low goblet cell numbers (<110). The majority of animals (12/16) from the control group (Fig-7.2) had high numbers (≥110) of goblet cells within the jejunum . In contrast, only 6/16 lambs from the *in utero* antigen-exposed lambs (Fig-7.2) had such high numbers (≥110) of goblet cells.

DISCUSSION

Many intestinal nematode infections induce goblet cell hyperplasia and an increase in mucin production within the gut (Else & Finkelman, 1998). Among sheep chronically infected with *Trichostrongylus colubriformis,* goblet cell hyperplasia is a consistent feature irrespective of the parasitic burden within the gut (Angus & Coop, 1984). The control lambs in the present study had a goblet cell response in the jejunum identical to that reported in literature. In the control lambs the goblet cells were consistently larger, had darker intensity of staining and were found clustered together in large numbers. In direct contrast, the *in utero* antigen-exposed lambs failed to exhibit similar goblet cell responses to the live larval challenge. These animals had goblet cells with smaller cell volumes, tended to stain with lower intensity and were found in lesser numbers within the epithelium. The number of goblet cells per microscopic field was significantly higher in the control group of lambs in comparison with the *in utero* antigen-exposed lambs. It thus appears that *in utero* antigenic exposure to the soluble third stage larval antigen was able to curtail the goblet cell response to the parasite in postnatal life.

At least part of the goblet cell response to nematode infections in the gut is thought to be under the control of a $CD4^+$ Th2 type response. Hypothymic rats infected with *Nippostrongylus brasiliensis* fail to exhibit a goblet cell response (Ishikawa *et al.*, 1994). Among *Trichinella spiralis* infected mice, goblet cell hyperplasia occurs eight days after infection when expulsion of the parasite occurs from the gut. At this time, lymphocytes from the draining MLN show a predominantly Th2 type response characterised by enhanced *in vitro* production

of IL-5 but not of interferon γ (Ishikawa *et al.*, 1997). IL-5 levels in the tissue were not assayed in the present study to substantiate whether there had been a polarised Th2 response. However, IL-2 and interferon γ levels, indicators of a Th1 polarised immune response, were quantified in the MLN. In the MLN of control lambs the IL-2 response was substantially higher, while the interferon γ levels were marginally higher in comparison with their respective levels in the *in utero* antigen-exposed lambs. Thus, the control lambs had marked goblet cell hyperplasia even though there were increased levels of interleukins suggestive of a Th1 type immune response in the MLN. The results of the present study contrast with those of Ishikawa *et al.* (1997) possibly because their results were based on *in vitro* studies. Perhaps this is a further suggestion that Th1 and Th2 responses are not quite as clear cut as their proponents argue.

The significantly reduced goblet cell response observed in the jejunum of *in utero* antigen-exposed lambs in comparison with control lambs could be partly explained based on the TNF-α levels in the jejunum. The *in utero* antigen-exposed lambs had significantly higher levels of TNF-α within the jejunum in comparison with control lambs. TNF-α has been shown to modulate the goblet cell response both *in vitro* and *in vivo*. HT29- N2 a colonic cancer cell line readily differentiates into goblet cells when grown in a specialised galactose containing medium. However, the addition of TNF-α to this media can retard the differentiation of this cell line into mucin producing goblet cells (Hamada *et al.*, 1991). *In vivo* evidence on the effect of TNF-α on goblet cell response in the gut comes from the investigation of Arnold *et al.* (1993). They investigated mucus production by goblet cells in the gastrointestinal tract following *Salmonella typhimurium* infection using a ligated ileal loop model in mice. *Salmonella* infection resulted in a decrease of almost 50% in the number of goblet cells and a concurrent increase in the TNF-α levels in comparison with uninfected animals. Arnold *et al.* (1993) also demonstrated that treatment of mice with antibody to TNF-α before *Salmonella* challenge minimised pathological changes in the gut and returned goblet cell numbers to levels comparable with those observed in uninfected control mice. It is likely that the expression of high levels of TNF-α within the jejunum of *in utero* antigen-exposed lambs could have curtailed the

maturation and differentiation of goblet cells from the pluripotent stem cells in the gut. The markedly curtailed goblet cell response in the jejunum of *in utero* antigen-exposed lambs could in its turn, have been one of the factors contributing to the marginally higher worm count in these lambs.

The physiochemical character of the mucus produced by the goblet cells could have also been different among the two groups of lambs. Further investigation would be required to clarify this point.

EOSINOPHILS

INTRODUCTION

Eosinophils are derived from the bone marrow and, although found in the peripheral circulation, they are primarily tissue dwelling cells. The eosinophil is distinguished by characteristic granules, which stain with acidic dyes such as eosin.

Eosinophils are regarded as proinflammatory granulocytes with roles in protection against parasitic infection, allergic reactions and chronic inflammatory diseases (Martin *et al.*, 1996). Several factors have been identified as chemotactic for eosinophils and are responsible for their recruitment into reaction sites. These chemotactic factors include platelet activating factor, histamine, leukotriene B4 and certain complement components (Wershil & Walker, 1992). At the reaction site, a number of stimuli can cause eosinophil degranulation and the subsequent release of both preformed as well as newly synthesised mediators, which can have multiple biological effects.

A striking feature of the degranulation phenomenon is the marked deposition in surrounding tissues of granule proteins which is often evident even in the presence of relatively small numbers of intact eosinophils (Martin *et al.*, 1996). However, the mechanisms of eosinophil degranulation *in vivo* are still poorly understood. Eosinophil degranulation is initiated and regulated by a multitude of factors including some which primarily stimulate the cells (eg. immunoglobulins like secretory IgA and lipid mediators), priming agents (eg. Cytokines IL-3, IL-5 and GM-CSF) and cellular adhesion molecules (Mac-1) (Martin *et al.*, 1996; Wershil & Walker, 1992). Preformed proteins that are released from eosinophils on degranulation include major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin, the first three of which are potent toxins for helminths and bacteria. They are also strongly implicated as mediators of the pathological changes observed in asthma and other eosinophil-associated diseases (Martin *et al.*, 1996). Eosinophil degranulation may also initiate further sequences of biochemical changes. For example, in addition to its toxic properties, major basic protein activates platelets, neutrophils, mast cells and basophils, the latter two of which release histamine. Activation of eosinophils also results in the *de novo*

synthesis of a number of factors which are subsequently released into the reaction site during degranulation. These include toxic oxygen radicals, leukotreines, platelet activating factors, cytokines such as TGF-α and β, IL-1 α and GM-CSF (Martin *et al.*, 1996; Wershil & Walker, 1992).

A number of factors modulate the activity of eosinophils. Among these IL-3, IL-5 and GM-CSF are the major cytokines responsible for the survival, differentiation and activation of eosinophils. These cytokines exert autocrine effects on eosinophils, by delaying apoptosis, thereby intensifying the eosinophil-mediated inflammatory response (Martin *et al.*, 1996).

Eosinophil infiltration and the deposition of eosinophil granular proteins appears to be intimately associated with the pathology of a number of clinical conditions. Tissue eosinophils are considered to be hallmarks of clinical conditions like eosinophilic gastroenteritis and celiac disease (Wershil & Walker, 1992). Eosinophil degranulation is thought to be responsible for the tissue damage observed in these conditions. Eosinophil infiltration has also been strongly correlated with T cell activation and specific cytokine production in several inflammatory diseases (Martin *et al.*, 1996).

Eosinophil involvement in gastrointestinal nematode infection in sheep: Blood eosinophilia is a commonly observed feature of the sheep's response to nematode infections particularly with *Trichostrongylus colubriformis* (Douch *et al.*, 1996b). A marked rise in blood eosinophil counts of *Trichostrongylus colubriformis*-infected lambs coincides with the start of the decline of the faecal egg count (Buddle *et al.*, 1992). The magnitude of the eosinophil response and the extent of nematode resistance are under genetic control (Buddle *et al.*, 1992). On the basis of the correlation between eosinophils and response to nematode infections, blood eosinophil counts have been one of the phenotypic markers used in the selective breeding of nematode resistant animals (Douch *et al.*, 1996b). Following *T. colubriformis* infection, sheep selected on the basis of low faecal egg counts tend to have higher eosinophil counts (high responders) while those with high faecal egg counts have a much lower eosinophilic response (low responders) (Buddle *et al.*, 1992; Stevenson *et al.*, 1994).

In sheep vaccinated with the irradiated, third stage larvae of *Trichostrongylus colubriformis*, the blood eosinophil responses were initially

similar in both high and low responder strains. However, following challenge of these immune animals with live larval parasites, the high responders had elevated eosinophil numbers in the blood (Rothwell *et al.*, 1993).

Pernthaner *et al.* (1995) have observed that in Romney lambs, genetically resistant to gastrointestinal nematode infections, the eosinophil counts in the peripheral blood began to increase one week earlier in comparison with the response of susceptible lambs after *T. colubriformis* infection

A number of investigators have demonstrated that a significant inverse correlation exists between blood eosinophil count and the faecal egg count of *Trichostrongylus colubriformis*-infected sheep (Buddle *et al.*, 1992; Douch *et al.*, 1996b). Hohenhaus *et al.* (1998) have observed that, in sheep carrying a mixed nematode infection of *T. colubriformis* and *Haemonchus contortus,* high eosinophilia in blood correlated with low faecal egg count. They have postulated that the retention of normal levels of circulating eosinophils is associated with resistance to stress (Hohenhaus *et al.*, 1998).

The significance of blood eosinophilia in *T. colubriformis*-infected sheep and of a negative correlation between eosinophil levels and faecal egg count has been questioned. Douch *et al.* (1996b) proposed that the underlying genetic relationships between blood eosinophil numbers and faecal egg count only become apparent in those animals in which eosinophil counts are elevated. Pernthaner *et al.* (1995) did not observe a significant correlation between the faecal egg count and the eosinophil count in *Trichostrongylus*-resistant lambs. However, in susceptible lambs, a significant correlation was found between faecal egg count and eosinophil counts at some sampling times. Based on this Pernthaner *et al.* (1995) have suggested that eosinophils may not have a direct role in mediating resistance to this nematode. In agreement with this conclusion, Huntley *et al.* (1995) did not observe any difference in the number of circulating eosinophils between control sheep and goats and an experimental group harbouring a mixed infection of *Trichostrongylus vitrinus* and *Teladorsagia circumcincta.*

Although eosinophilia occurs in nematode-infected ruminants, the nature of the relationship between blood eosinophil count and the effector function in the gut remains to be clearly established (Miller, 1996). A consensus that seems

to be emerging is that eosinophilia is associated with the expression of resistance to nematodes in sheep rather than being an indicator of the presence of nematode infection (Buddle *et al.*, 1992; Pernthaner *et al.*, 1995).

A most striking feature of gastrointestinal nematode infections is an increase in the number of eosinophils and mast cells within the mucosa of the Gastrointestinal tract (Gill *et al.*, 1993; Miller, 1984). Among lambs that are genetically resistant to *Haemonchus contortus*, exposure to the nematode results in a marked eosinophilic infiltration of the abomasum in comparison to randomly bred lambs (Gill *et al.*, 1993). The same workers also observed that both tissue eosinophilia and resistance to *Haemonchus* were abolished in lambs depleted of $CD4^+$ T cells. Gill *et al.* (1993) proposed that the generation of eosinophilic responses in nematode-infected sheep is controlled by factors produced by $CD4^+$ T cells.

Pfeffer *et al.* (1996) dosed sheep orally with 10,000 larvae of *Trichostrongylus axei* for eight weeks. After this exposure they assessed the extent of cellular infiltration and worm burden in the abomasum. There was a significant increase in the density of eosinophilic infiltration of the abomasal mucosa in these sheep in comparison with uninfected animals.

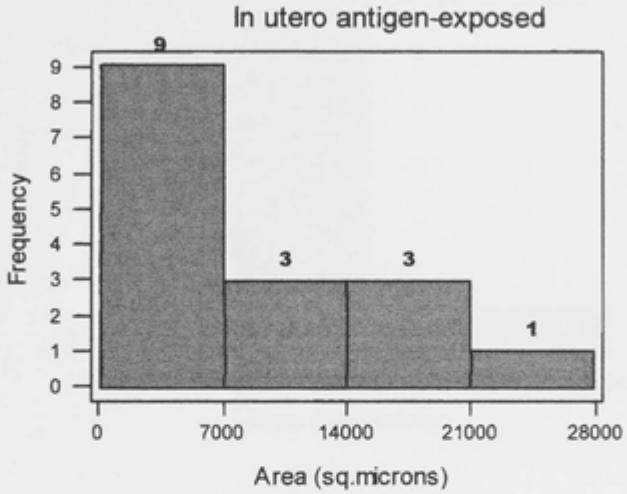
Eosinophils from the gastrointestinal tract of sheep are capable of IL-5 expression. It appears that IL-5 might be important in regulating the autocrine and paracrine activation of effector cells involved in the immune expulsion of parasites. Bao *et al.* (1996) have observed that the number of eosinophils positive for IL-5 mRNA in the lamina propria of the jejunum of immune sheep was about five times greater than that of naïve sheep challenged with a single dose of 30,000 third stage larvae of *T. colubriformis*. These eosinophils represented about 80 % of all IL-5 $mRNA^+$ cells. There was only a marginal increase in the number of IL-5 $mRNA^+$ eosinophils in the mesenteric lymph node. There were very few eosinophils in the Peyer's patches and no observed differences between immune and control animals (Bao *et al.*, 1996).

The study of Douch and Morum (1993) indicated that the extent of eosinophil infiltration in the gastrointestinal tract was unlikely to be age dependent. Three groups of Romney sheep, reared worm-free in pens from birth until 4, 16 or 28 weeks of age were permitted to graze nematode infected pasture

Table- 8.1 : Total positive area – Eosinophils

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	8399 ± 1711	7347 ± 1220	NS
Mesenteric lymph node	4759 ± 592	3935 ± 589	NS
Ileal Peyer's patch	1541 ± 271	2735 ± 636	P=.05

Fig-8.2: Frequency distribution of eosinophils in the jejunum



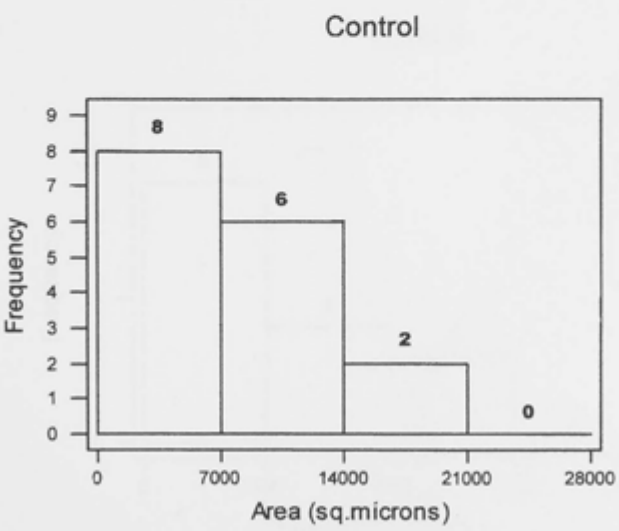
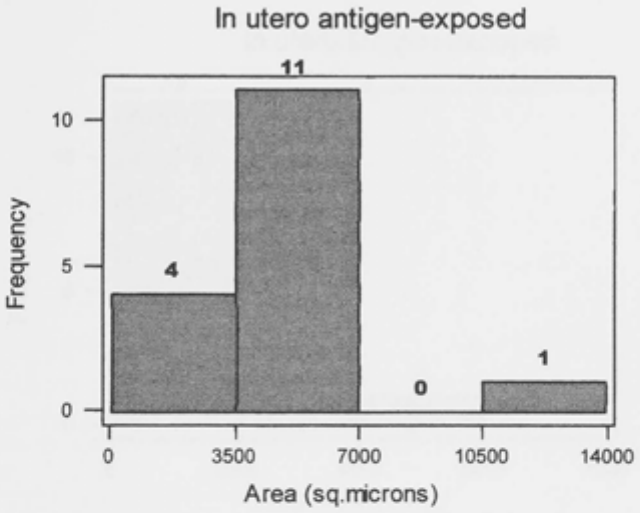


Fig-8.3: Frequency distribution of eosinophils in the MLN



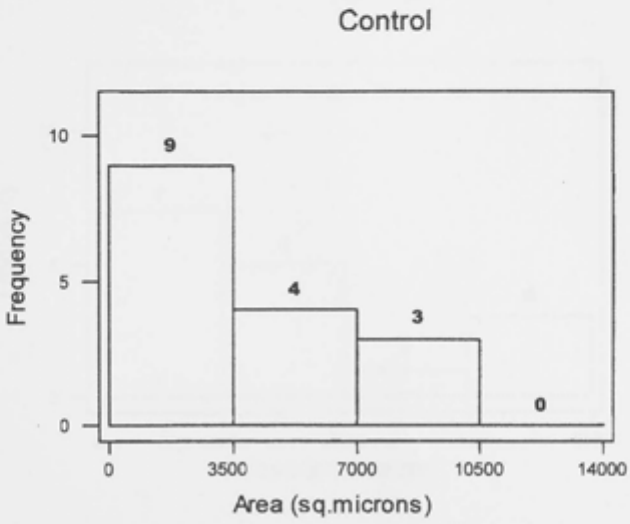
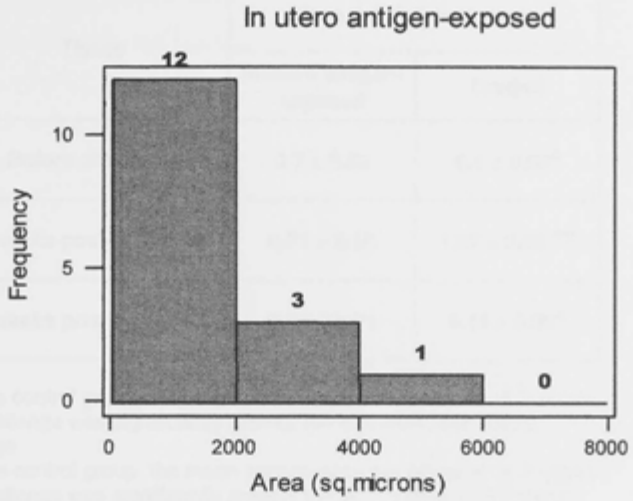


Fig-8.4: Frequency distribution of eosinophils in the IPP



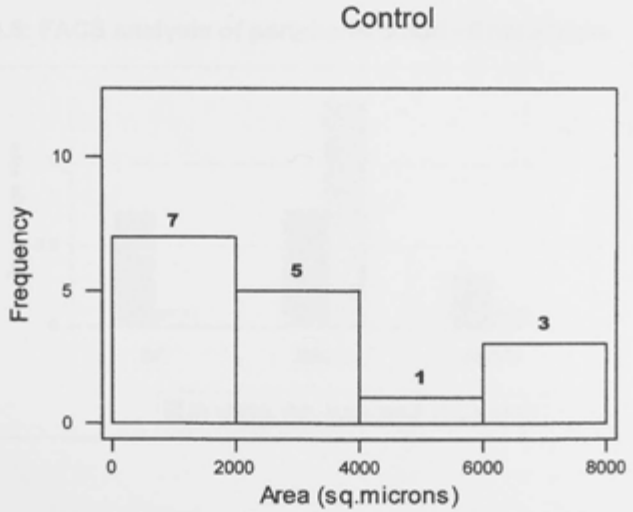


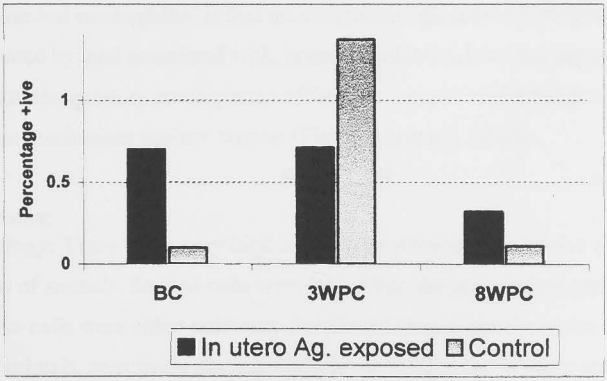
Table-8.5 : FACS analysis of peripheral blood- Eosinophils

Tissue	Mean ± S.E. (percentage)	
	In utero antigen-exposed	Control
Before challenge	0.7 ± 0.55	0.1 ± 0.06[A]
3 weeks post-challenge	0.71 ± 0.56	1.37 ± 0.63 [A,B]
8 weeks post challenge	0.32 ± 0.21	0.11 ± 0.06[B]

A: In the control group, the mean percentage of eosinophils at 3 weeks post challenge was significantly greater (p=.030) than that before challenge

B: In the control group, the mean percentage of eosinophils at 3 weeks post challenge was significantly greater (p=.031) than that at 8 weeks post challenge

Fig-8.6: FACS analysis of peripheral blood - Eosinophils



for four weeks after which they were returned to the pens and slaughtered after a further interval of four weeks. Whilst the frequency of eosinophils in the gastrointestinal mucosa did not vary with the age of either infected or control sheep, eosinophil numbers in the mucosa of the infected lambs were significantly higher than those in the uninfected animals (Douch & Morum, 1993).

There are indications that eosinophils *per se* do not have a direct 'effector' action against intestinal nematodes although an indirect role cannot be discounted (Else & Finkelman, 1998). Naïve sheep exposed to a mixed infection of *Trichostrongylus vitrinus* and *Teladorsagia circumcincta* did not manifest an increase in the eosinophilic infiltration of the jejunum (Huntley *et al.*, 1995). Likewise, immune ewes and does challenged with the same mixed nematode infection did not have significant eosinophilic infiltration of the jejunum (Huntley *et al.*, 1995). Trickle infection (3000 third stage larvae twice weekly for 7 weeks) with *T. colubriformis* and subsequent challenge with a single bolus infection of 30,000 larvae evoked eosinophilia in only some lambs (McClure *et al.*, 1998).

Clearly any consensus in the literature on the question of whether eosinophils play an effector role in the gastrointestinal tract of nematode infected sheep is lacking. An alternative hypothesis to explain the association between infection and eosinophilia is that the eosinophil-IgE-mast cell response stimulated by, and associated with, helminth infection is an immunopathological or immunoregulatory consequence of the host-parasite relationship rather than an effector mechanism against worms (Finkelman *et al.*, 1991).

RESULTS

Jejunum:

Histology: There was a very high intensity of staining of positive cells in both groups of animals. Stained cells were larger than the surrounding lymphocytes. Positive cells were either randomly distributed throughout the entire section or, in other animals, selectively clustered within the villi. In the *in utero* antigen-exposed group there were fewer animals (5/16) with extensive infiltration of the villi. In contrast, the majority of animals (10/16) from the control group had marked eosinophilic infiltration of the core of the villi. In some cases, the positive cells were seen clustered towards the apical end of the villi.

Image analysis: The total positive area staining for eosinophils was not

significantly different (statistically) between the two groups of animals. The mean positive area was marginally higher in the *in utero* antigen-exposed group of animals in comparison with control lambs (Table-8.1).

The frequency distribution of animals when the area occupied by eosinophils in the jejunum was divided into four equal ranges revealed only slight differences between the two groups. The number of animals with a positive area >14,000sq.μ was marginally higher (4/16) in the *in utero* antigen-exposed group (Fig-8.2) in comparison with the control group(2/16).

Mesenteric lymph node

Histology: The positive cells tended to be larger than the surrounding lymphocytes. They were either found in patchy clusters (4-20 cells) or as randomly distributed cells between the trabeculae of the medullary region. The intensity of staining could be graded as either extremely dark staining or as pale. In the MLN of individual animals, the cells stained either with low or high intensity but seldom with a mixture of both. The majority of animals (10/16) from the *in utero* antigen-exposed group had lighter staining cells. In direct contrast, the majority of animals (9/16) from the control group had darker staining eosinophils in the MLN.

Image analysis: Although the difference was not statistically significant, the mean positive area staining for eosinophils was marginally higher (18 %) in the MLN of *in utero* antigen-exposed lambs in comparison with control animals (Table-8.1).

The frequency distribution of animals when the area occupied by eosinophils was divided into four equal ranges revealed distinct differences between the two groups. In the *in utero* antigen-exposed group the majority of animals (11/16) had a moderate range (>3500 and <7000 sq.μ) of positive area (Fig-8.3). In contrast, only 25 % of control animals (4/16) fell within the same range (>3500 and <7000 sq.μ) (Fig-8.3). In the control group the majority of animals (9/16) had a low positive area (>0 and <3500 sq.μ) while only 25 % of animals from the *in utero* antigen-exposed group had the same range (>0 and <3500 sq.μ) of positive cells (Fig-8.3).

Ileal Peyer's patch:

Histology: The cells were invariably dark staining and tended to be larger than the surrounding lymphocytes. In the *in utero* antigen-exposed lambs there were fewer cells infiltrating into the villi. In contrast, control animals had larger number of positive cells clustered within the villi. Among *in utero* antigen-exposed lambs the number of positive cells found in the interfollicular regions was considerably less than in the control group which had large numbers of eosinophils in close apposition with the surrounding lymphocytes of the interfollicular region. In both groups of animals, a few positive cells were also found in close proximity to the connective tissue capsule of the follicles as well as near the muscularis mucosa.

Image analysis: The mean area occupied by eosinophils in the IPP was significantly greater ($p=.05$) in the control group of animals than in the *in utero* antigen-exposed lambs (Table-8.1).

The frequency distribution of animals, when the area occupied by eosinophils was divided into four equal ranges revealed distinct differences between the two groups. In the *in utero* antigen-exposed group 75 % of the lambs (12/16) had a low range (>0 and <2000 sq.μ) of positive cells within the IPP (Fig-8.4). In contrast, there were only 7/16 animals with the same range (>0 and <2000 sq.μ) of positive cells (Fig-8.4). Only one animal among the *in utero* antigen-exposed group had a positive area >4000 sq.μ. Among control lambs 25 % of the animals had a positive area >4000 sq.μ.

FACS analysis of peripheral blood:

Before larval challenge, the mean level of eosinophils in the peripheral circulation of *in utero* antigen-exposed lambs was higher (but not statistically significant) in comparison with control lambs. There were no significant changes in the dynamics of eosinophil levels in the peripheral circulation of *in utero* antigen-exposed lambs at any of the three points of time (Table-8.5 and Fig-8.6). The only change observed after challenge of these lambs was a marginal drop in mean percentage level at eight weeks post-challenge. In contrast with this lack of variation, in the control group of lambs, there was a significant increase ($p=.03$) in the mean percentage level of eosinophils three weeks after challenge in comparison with the pre-challenge level. This elevated level of eosinophils was

not maintained in the control group and by eight weeks post-challenge there was a significant drop (p=.031) in the mean level in comparison with that observed at three weeks post challenge (Table-8.5 and Fig-8.6).

DISCUSSION

Eosinophils are regarded as proinflammatory granulocytes that play an important role in protection against parasitic infections (Martin *et al.*, 1996). It was observed that the antigenic-exposure of the foetal gut could significantly alter the eosinophilic response in both the peripheral circulation and the gastrointestinal tract.

Before larval challenge the mean level of eosinophils in the peripheral circulation of *in utero* antigen-exposed lambs was substantially higher (not statistically significant) in comparison with the control lambs. It is possible that the parasitic antigen administered via the oral route, *in utero* could have stimulated the generation of eosinophils from bone marrow precursors. Haig *et al.* (1995) have shown that ten days after a primary infection with the nematode parasite *Telodorsagia circumcincta* the frequency of eosinophil colony forming progenitor cells within the bone marrow is considerably higher in comparison with uninfected animals. It does not appear that the increased number of eosinophils detected in the peripheral circulation of *in utero* antigen-exposed lambs before larval challenge represented granulocytes that were actively migrating into the gut. The evidence for this conclusion is provided by the kinetics of the eosinophilic response three weeks after larval challenge. Among the *in utero* antigen-exposed lambs eosinophil levels in the peripheral circulation did not undergo any change after larval challenge. This indicates that depletion of eosinophils from the peripheral circulation with homing into the gut tissue was unlikely. In direct contrast, control lambs had a significantly elevated eosinophilic response in the peripheral circulation, three weeks after larval challenge in comparison with the prechallenge levels. Eosinophilia in the peripheral circulation seems to be a consistent feature after primary exposure of foetal and perinatal lambs' gut to *Trichostrongylus colubriformis* larval antigen and live larvae respectively. The only differentiating feature was that the magnitude of the eosinophilia was considerably lower in the peripheral circulation of *in utero* antigen-exposed lambs in comparison with the control

lambs. Thus it appears that the gut immune system of foetal lambs at 100 days of age has reached a developmental stage similar to that of the adult sheep in terms of generation of an eosinophilic response in the peripheral circulation after a primary oral antigenic exposure. It appears that the primary infection of control lambs with the larvae caused significant activation of the bone marrow precursors resulting in a significant eosinophilia in the peripheral circulation.

The marked eosinophilia observed in the peripheral circulation of control lambs three weeks after larval challenge correlated negatively with the occurrence of a lower faecal egg count during the third and eighth week post challenge. A number of other investigators have also demonstrated that peripheral blood eosinophilia encountered after *Trichostrongylus colubriformis* infection is associated with a lower faecal egg count (Buddle *et al*., 1992; Douch *et al*., 1996b).

Eight weeks after larval challenge, both groups of lambs exhibited a drop in the eosinophil levels in the peripheral circulation in comparison with the levels detected three weeks post challenge. The extent of depletion of eosinophils from the peripheral circulation was significantly higher among control lambs while being only marginal in the *in utero* antigen-exposed group. It possible that the eosinophils depleted from the peripheral circulation could have homed into the gut wall at this time. The *in utero* antigenic-exposure was able to alter the dynamics of depletion of eosinophils from the peripheral circulation at this point of time.

The IPP was the gut tissue in which the eosinophilic response was significantly altered by the *in utero* antigenic-exposure. The mean area staining for eosinophils in the IPP was significantly greater in the control group of animals in comparison with the *in utero* antigen-exposed lambs. The control lambs had large numbers of positive cells clustered within the villi and the interfollicular region. Among *in utero* antigen-exposed lambs there were fewer eosinophils within the villi and the interfollicular region of the IPP. This could have resulted from two possibilities. In the case of *in utero* antigen-exposed lambs a significant number of eosinophils could have migrated away from the IPP into other sites like the MLN and the jejunum. The significant difference could also have resulted from the increased immigration of eosinophils into the

IPP of control lambs. The results of the present study contrast with the findings of Bao *et al.* (1996). They did not observe any change in the number of IL-5 mRNA positive eosinophils in the IPP of naïve or immune sheep that were challenged with 30,000 larvae of *Trichostrongylus colubriformis*. While Bao *et al.* (1996) used the same dose of larvae for challenge as in the present study there were other important differences from the present study which could have accounted for the absence of an eosinophilic response in the IPP. The histological response in the gut was based on examination of gut tissue three days after larval challenge while in the present study this was done on tissues collected after 8 weeks. Furthermore, Bao *et al.* (1996) used adult sheep in their investigations while in the present study young lambs of 3-4 months of age were used.

Thus *in utero* antigenic exposure followed by re-exposure to the homologous antigen in postnatal life caused significant changes in the numbers of eosinophils infiltrating the IPP. The eosinophils from the IPP of *in utero* antigen-exposed lambs may have homed into the jejunum and MLN. This migration was suggested by the marginally higher area staining for eosinophils within the jejunum and MLN of *in utero* antigen-exposed lambs in comparison with control lambs. In addition to eosinophils, a significant number of $CD4^+$ and $CD8^+$ T lymphocytes were also depleted from the IPP of *in utero* antigen-exposed lambs and these seem to have homed into the jejunum and MLN. These T lymphocytes could have actively secreted cytokines like IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor which might have activated and mobilised eosinophils from the IPP and recruited them into the jejunum and MLN. The marginally elevated levels of eosinophils within the jejunum could have been partly responsible for the increased incidence of clinical symptoms such as diarrhoea which occurred among *in utero* antigen-exposed lambs. There is increasing evidence that T cells play an important role in the recruitment of eosinophils to the respiratory tract in clinical conditions such as asthma. The immunopathological changes are thought to be driven and maintained by a subset of chronically activated memory T cells. On sensitisation with allergens these T cells rapidly home into the respiratory tract where they actively secrete an array of cytokines including IL-3, IL-5 and granulocyte-macrophage colony

stimulating factor which recruit and mobilise eosinophils for subsequent mucosal damage (Garlisi *et al*., 1995; Kon & Kay, 1999).

The increased number of eosinophils within the jejunum of *in utero* antigen-exposed lambs was not matched by a concurrent histological response. The eosinophils within the jejunum of *in utero* antigen-exposed lambs were randomly distributed throughout the whole section. In marked contrast, the majority of animals from the control group of lambs had marked infiltration of eosinophils into the core of the villi. The histological features of the jejunum of *in utero* antigen-exposed lambs suggest two possibilities. The first is that the infiltrating eosinophils have already carried out their effector functions and subsequently dispersed throughout the tissue. The second possibility is that the eosinophils are yet to recognise the parasite in gut and carry out their effector function. In contrast, the eosinophils from the control lambs seem to be actively recognising the infection and carrying out an effector function. This interpretation was also supported by the marginally lower worm count in the gut of control lambs. Thus, marked reduction in the number of eosinophils from the IPP coupled with an increased eosinophilic infiltration of the jejunum and MLN might be detrimental to the efforts of the lamb's defence mechanisms in lowering the worm burden in the gut.

TUMOR NECROSIS FACTOR-α

INTRODUCTION

Tumor necrosis factor α (TNF-α) can be produced by virtually all cells upon activation (Debard *et al.*, 1999). It is produced by activated macrophages, often in response to parasitic, bacterial or viral infections (Barbara *et al.*, 1996; Egan *et al.*, 1994b). TNF-α is initially synthesized as a membrane bound precursor protein (Debard *et al.*, 1999; Korner *et al.*, 1997). It is cleaved from the cell surface within hours of its appearance, by a highly specific metalloproteinase (Eigler *et al.*, 1997). When it is cleaved from the cell surface this soluble monomer aggregates into a homotrimer *in vivo* to give rise to the biologically active form (Barbara *et al.*, 1996; Egan *et al.*, 1994b). Many of the biological effects of TNF-α are mediated through the cross linking of two distinct receptors- TNFR I (p55) and TNFR II (p75) (Brockhaus *et al.*, 1990; Debard *et al.*, 1999). TNFR II is often more abundant on cells of haemopoietic lineage whereas TNFR I, although it tends to be ubiquitous, does not exist on erythrocytes and unstimulated T cells (Brockhaus *et al.*, 1990; Hohmann *et al.*, 1989). Each of these receptors mediates different biological activities. The engagement of the p55 receptor mediates cytotoxicity, fibroblast proliferation and prostaglandin synthesis. The p75 receptor, which binds TNF-α with a higher affinity, is responsible for T cell proliferation and cytokine release (Tartaglia *et al.*, 1991).

It has been previously assumed that the principal activities of TNF were mediated by the soluble form only. However, it is now clear that the membrane-bound TNF-α can also exhibit potent cytotoxic and tumoricidal activities (Kriegler *et al.*, 1988). Furthermore, membrane TNF has been demonstrated to be an important ligand for TNFR II and the parallel engagement of TNFR I and II during direct cell-cell contact leads to dramatically enhanced cytotoxic function (Grell *et al.*, 1995).

TNF-α is a hormone-like peptide that can either enter the blood stream to alter the biology of distant tissues or behave as a paracrine mediator acting locally. It may be regarded as a true pleiotropic cytokine with numerous biological effects including cytotoxic, anti-infective, growth modulating and cellular differentiation properties (Barbara *et al.*, 1996; Vassalli, 1992).

TNF-α and the gastrointestinal tract: The expression of TNF-α in the gut is generally associated with a proinflammatory response. Inflammatory bowel disease in humans, most notably Crohn's disease and ulcerative colitis, is characterised by elevated levels of TNF-α in the gut (Noguchi *et al.*, 1998; Woywodt *et al.*, 1999).

There have been a number of reports of changes in TNF-α in response to parasitic infections of the gut. In *Trichinella spiralis* infection of rats, the TNF-α induced in the cells of the small intestine is thought to accumulate in the MLN which act like a sink. This conclusion was based on a continuous assay of induced cytokines appearing in the intestinal lymph entering the afferent lymphatic vessels of the MLN and the thoracic duct lymph which is efferent from the MLN (Ramaswamy *et al.*, 1996).

Trichinella spiralis infection induces substantial amounts of TNF-α in the murine intestine resulting in an enteropathy characterised by villous atrophy, crypt hyperplasia and an increase in the number of mitotic figures. However, TNF-α gene knockout mice displayed markedly reduced pathology in the intestine compared with normal mice without suffering any decreased capacity to expel the parasite (Lawrence *et al.*, 1998). This work strongly suggested that TNF-α is involved in the development of pathological changes during nematode infections.

Within the ileal mucosa of neonatal calves infected with *Cryptosporidium parvum* the expression of TNF-α is down-regulated. Once the animals recovered from the infection the normal level of cytokine expression in the intraepithelial T lymphocytes was re-established (Wyatt *et al.*, 1997).

In *Toxoplasma gondii* infected mice TNF-α production is upregulated in the lamina propria of the small intestine (Liesenfeld *et al.*, 1999). In addition, the same workers were able to demonstrate that treatment of infected mice with anti TNF-α antibodies prevented necrosis in the intestinal villi and mucosal cells and prolonged the survival of infected animals. Treatment with the antibody also inhibited the expression of inducible nitric oxide synthase (iNOS) in infected mice. They suggested that nitric oxide induced by TNF-α through the activation of iNos is a critical mediator of intestinal pathology.

TNF-α and the gastrointestinal tract of sheep: Monoclonal antibody to ovine TNF-α has been produced and characterised (Egan *et al.*, 1994b). However, published evidence of it's significance in the ovine gut seems to be very limited.

In sheep naturally infected with Johne's disease the distal ileum contained significantly elevated levels of mRNA for TNF-α (Alzuherri *et al.*, 1996). Concurrent histological examination of the gut revealed villous atrophy and disruption of the normal mucosal architecture. Marked infiltration of macrophages into the lamina propria was also evident. Alzuherri *et al.* (1996) also observed that a number of uninfected animals also expressed TNF-α mRNA in the ileum, albeit at a lower level than infected animals. They concluded that even under normal physiological conditions, the intestine of grazing sheep could manifest some cytokine activity due to exposure to a vast array of antigens.

Begara-McGorum *et al.* (1998) experimentally infected neonatal lambs (<2 week old) orally with *Mycobacterium avium* subspecies *paratuberculosis* and investigated the immunological changes up to eight weeks post infection. They found that one of the most notable features was the significant up-regulation of TNF-α mRNA in the MLN. However, in contrast to the changes in the MLN there was a decrease in the TNF-α mRNA expression in the jejunal Peyer's patches in comparison with control lambs. In the ileal Peyer's patches the transcript for TNF-α was found only in one infected animal while four out of eight control animals had appreciable levels of expression of mRNA for TNF-α.

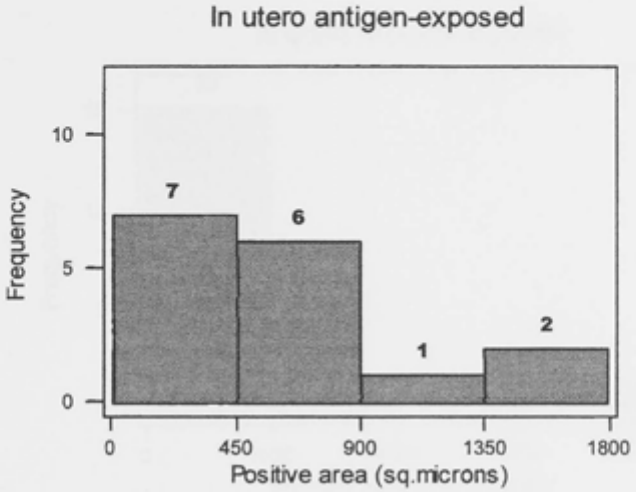
TNF and organogenesis: Evidence has been presented suggesting that TNF-α might play an important role in the organogenesis of lymphoid organs in mice. However, there have been considerable differences between various reports regarding the significance of TNF-α for lymphoid organ organogenesis.

Korner *et al.* (1997) have demonstrated that the normal development of the murine Peyer's patches is substantially dependent on TNF-α. The Peyer's patches in $TNF^{-/-}$ knockout mice were macroscopically smaller. Immunohistology demonstrated a complete loss of the defined T and B cell structures with intermixing of these cell types and a reduced number of B cells. The serum immunoglobulin isotype expression was indistinguishable from that seen in the wild type mice. Likewise, following immunisation with T-dependent antigens

Table- 9.1 : Total positive area – TNF- α

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	575 ± 106	356 ± 72	P=.05
Mesenteric lymph node	755 ± 501	422 ± 163	NS
Ileal Peyer's patch	640 ± 165	1224 ± 285	P=.045

Fig-9.2: Frequency of occurrence of TNF-α in the jejunum



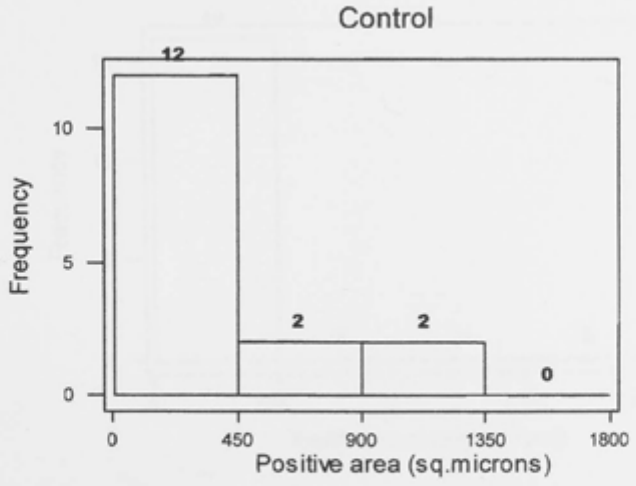
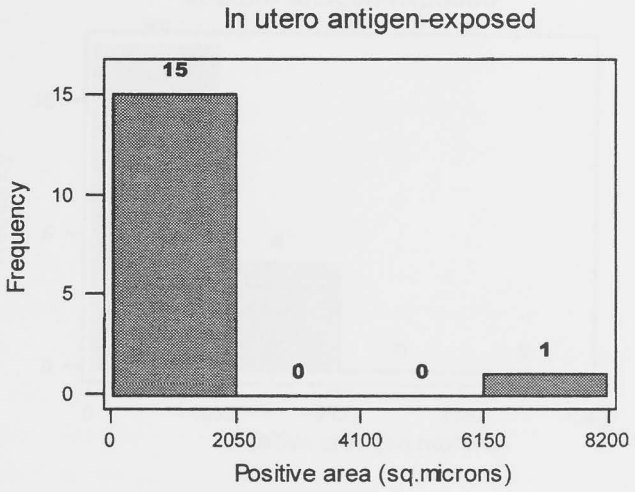


Fig-9.3: Frequency of occurrence of TNF-α in the MLN



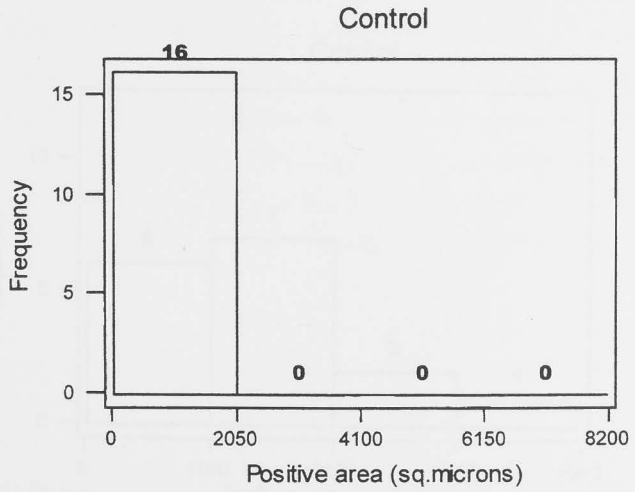
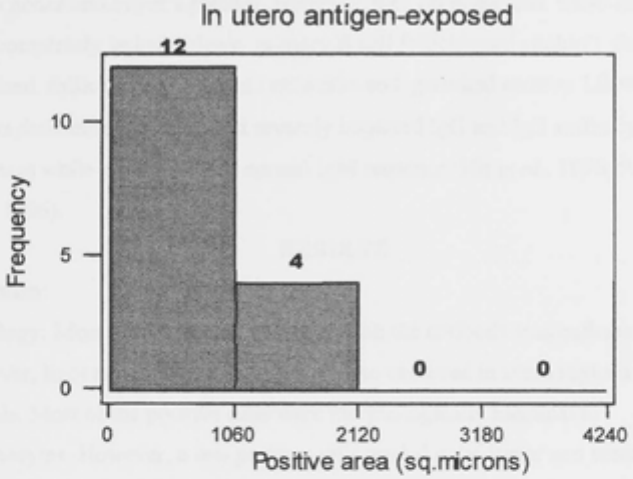
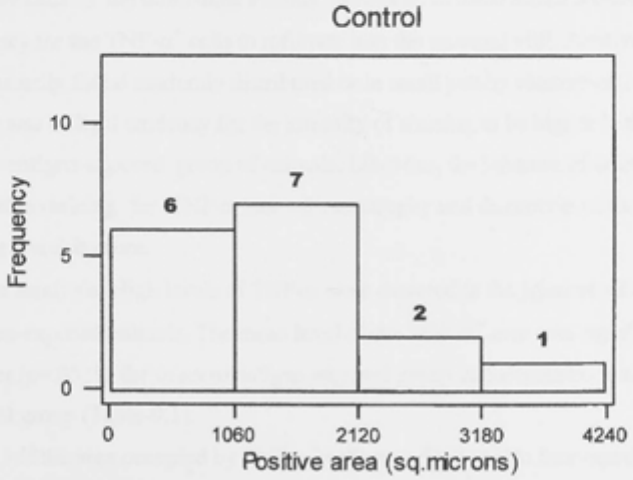


Fig-9.4: Frequency of occurrence of TNF-α in the IPP





there was no obvious impairment of the specific humoral immune response. However, contrary to this report, other workers (Pasparakis *et al.*, 1996; Pasparakis *et al.*, 1997) have reported that TNF-α knockout mice develop normal lymph nodes and Peyer's patches. However, they observed that these knock out mice completely lacked splenic primary B cell follicles and couldn't form organised follicular dendritic cell networks and germinal centres. Likewise, TNF-α deficient mice exhibited severely impaired IgG and IgE antibody responses while maintaining a normal IgM response (Hir *et al.*, 1996; Pasparakis *et al.*, 1996).

RESULTS

Jejunum:

Histology: Most of the staining observed with the antibody was cell-associated. However, light extracellular staining was also observed in some regions of a few animals. Most of the positive cells were morphologically identical to lymphocytes. However, a few positive cells tended to be larger and some of them had dendritic morphology. Most of the positive cells were localized to the lamina propria. In those jejunal sections in which cross sections of *Trichostrongylus* were observed to be embedded in the mucosa there was an absence of TNF-α positive cells in the immediate vicinity. However, in these animals there was a tendency for the TNF-α^+ cells to infiltrate into the mucosal villi. Positive cells were usually found randomly distributed or in small patchy clusters of 2-5 cells. There was a slight tendency for the intensity of staining to be higher in the *in utero* antigen-exposed group of animals. Likewise, the jejunum of animals with extensive staining for TNF-α had villous atrophy and disruption of the normal mucosal architecture.

Image analysis: High levels of TNF-α were detected in the jejunum of *in utero* antigen-exposed animals. The mean level of the TNF-α^+ area was significantly greater ($p=.05$) in the *in utero* antigen-exposed group in comparison with the control group (Table-9.1).

If the area occupied by TNF-α^+ cells was divided into four equal ranges and the frequency distribution of animals in each range was presented, distinct differences between the two groups became evident. In the *in utero* antigen-exposed group (Fig-9.2) there were only seven animals with very low levels (>0

and <450 sq.μ) of TNF-α. On the contrary, in the control group 12/16 animals (75 %) had very low levels (>0 and <450 sq.μ) of TNF-α in the jejunum(Fig-9.2). A majority of the animals (9/16) in the *in utero* antigen-exposed group had TNF-α levels >450 sq.μ. However, only 4/16 (25 %) animals in the control group had TNF-α >450 sq.μ.

Mesenteric lymph node:

Histology: Most of the observed staining was cell associated. However, extracellular staining was evident in some regions expecially when positive cells were distributed in large patches. The vast majority of positive cells were morphologically similar to lymphocytes. Occasionally, in certain regions, larger positive cells with dendritic morphology were also evident. The intensity of staining ranged from medium to low. The positive cells were found randomly distributed or as large clusters (>20 cells). No histological differences between the two groups were apparent.

Image analysis: Though not statistically significant, the mean level of TNF-α detected in the MLN tended to be higher in the *in utero* antigen-exposed group in comparison to the control group (Table-9.1).

When the area occupied by the TNF-α^+ cells was divided into four equal ranges the frequency distribution of animals between the two groups (Fig-9.3) was very similar.

Ileal Peyer's patch:

Histology: Almost all the staining observed for TNF-α was cell associated. Rarely, a small amount of extracellular staining was evident. Positive cells were almost exclusively lymphocytes. Large positive cells with dendritic morphology were only rarely observed. The intensity of staining of the positive cells ranged from medium to low. The TNF-α^+ cells found in the IPP stained more darkly in comparison with the jejunum and MLN where positive cells tended to be more lightly stained. The positive cells were found in large clusters (>20 cells) and were almost exclusively confined to the mucosa and the mucosal villi. Only on rare occasions were a few positive cells seen randomly distributed in the interfollicular region. The cells within the follicles were never positive for TNF-α. The intensity of staining seemed to be marginally higher in the control

group of animals. There were no other histological differences between the two groups.

Image analysis: High levels of TNF-α were detected in the IPP of the control group of animals. The mean level was significantly greater (p=.045) in the control group in comparison with that of the *in utero* antigen-exposed group (Table-9.1).

When the area occupied by TNF-α^+ cells in the IPP was divided into four equal ranges and correlated with the frequency distribution, distinct differences between the two groups became apparent. In the *in utero* antigen-exposed group (Fig-9.4) a majority of animals (12/16) had low levels (>0 and < 1060 sq.μ) of TNF-α^+ cells within the IPP. On the contrary, in the control group (Fig-9.4) there were only 6/16 animals with such a low range (>0 and <1060 sq.μ) of TNF-α in the IPP. In the *in utero* antigen-exposed group only 25 % (4/16) of animals had a TNF-α^+ area >1060 sq.μ in the IPP. However, in the control group the majority of animals (10/16) had a positive area >1060 sq.μ.

DISCUSSION

Most published literature emphasises the importance of macrophages as the major source of TNF-α (Barbara *et al.*, 1996; Eigler *et al.*, 1997; Vassalli, 1992). However, in the present study immunohistochemical staining of the jejunum, MLN and IPP revealed very few TNF-α cells with morphology that was typical of macrophages. Large amounts of TNF-α could still be detected in serial sections of jejunum, MLN and IPP, even when very few macrophages (CD14$^+$) and dendritic cells (CD1b$^+$ and CD1c$^+$) were present. The vast majority of TNF-α^+ cells were morphologically identified as lymphocytes. Activated lymphocytes, especially T cells, are also an important source of TNF-α (Vassalli, 1992). There were strong suggestions that many of the TNF-α^+ cells detected in the present study might be T lymphocytes. In serial sections of the jejunum and the IPP in which there were very few B lymphocytes (identified by the pan B lymphocyte marker CD45R), TNF-α^+ lymphocytes could be still detected in large numbers. Thus, it appears that among *Trichostrongylus*-infected sheep the major source of TNF-α within the gut is likely to be lymphocytes rather than macrophages.

In addition to cell-specific staining for TNF-α, a small amount of extracellular staining was also evident in the gut tissue of some animals. This could have been nonspecific staining. However, most of the extracellular staining observed was found in close association with TNF-α^+ cells. The extracellular staining thus might be specific and possibly represents the soluble form of the cytokine. TNF-α can exist in a soluble form or an unprocessed membrane bound form (Barbara *et al.*, 1996).

Marked induction of TNF-α was observed within the jejunum and the MLN of *in utero* antigen-exposed lambs in comparison with the control animals. The total area staining for TNF-α within the jejunum was significantly greater in the *in utero* antigen-exposed group in comparison with the control group of lambs. Likewise, the *in utero* antigen-exposed lambs had 44 % more TNF-α within the MLN in comparison with control lambs. The expression of TNF-α in the gut is generally associated with a proinflammatory response. Inflammatory bowel disease in humans, most notably Crohn's disease and ulcerative colitis, is characterised by elevated levels of TNF-α in the gut (Noguchi *et al.*, 1998; Woywodt *et al.*, 1999). The elevated TNF-α levels within the jejunum and MLN of *in utero* antigen-exposed lambs could have contributed to the adverse clinical symptoms such as diarrhoea observed in this study. Histologically, elevated levels of TNF-α were associated with villous atrophy and disruption of the normal mucosal architecture. Enteropathy induced by TNF-α is a characteristic feature in some infections of the gut. For example *Trichinella spiralis* infection induces substantial amounts of TNF-α in the intestine of mice, resulting in an enteropathy characterised by villous atrophy, crypt hyperplasia and an increase in the number of mitotic figures (Lawrence *et al.*, 1998). Among sheep naturally infected with paratuberculosis the distal ileum contained significantly elevated levels of mRNA for TNF-α. The concurrent histological findings included villous atrophy and disruption of the normal mucosal architecture (Alzuherri *et al.*, 1996).

The significantly elevated levels of TNF-α within the jejunum and the MLN were not associated with a low worm count in the gut of *in utero* antigen-exposed lambs. Other investigators have also reported that TNF-α induced during

parasitic infection of the gut might be detrimental to the recovery from the infection. Among *Toxoplasma gondii* infected mice TNF-α production is upregulated in the lamina propria of the small intestine. If anti-TNF-α antibody is administered during the course of infection with this parasite, necrotic lesions in the intestine can be prevented and the survival time of the infected mice prolonged (Liesenfeld *et al.*, 1999). Likewise, among TNF-α gene knockout mice there is marked reduction in the severity of intestinal pathology following *Trichinella spiralis* infection. At the same time there is no detrimental effect on the capacity of these mice to expel the parasite (Lawrence *et al.*, 1998).

Thus, the gut mucosal immune system of the foetal lamb at 100 days of gestation seems to have reached a developmental stage where it can be modulated by antigenic exposure and, on re-exposure to the homologous antigen in postnatal life, can respond with massive induction of TNF-α in the gastrointestinal tract.

A question that arises in this context concerns the nature of mechanisms involved in the substantial amplification of the TNF-α response in the jejunum and MLN of *in utero* antigen-exposed lambs. During response to re-exposure, a larger number of resident lymphocytes and macrophages in the jejunum and MLN could have been activated resulting in the induction of TNF-α. Evidence to support this possibility is lacking in the present study. It is also possible that a large number of these TNF-α^+ cells might have migrated in from other regions of the gut. There is evidence to support this possibility in the present study. The mean positive area staining for TNF-α within the IPP was significantly higher in the control group of lambs in comparison with the *in utero* antigen-exposed lambs. Among *in utero* antigen-exposed lambs when the TNF-α levels in the jejunum and MLN were high the corresponding level in the IPP was low. There were very few positive lymphocytes within the IPP mucosa of *in utero* antigen-exposed lambs. Thus it appears that a substantial number of these TNF-α lymphocytes may have migrated from the IPP and homed into the jejunum and MLN of *in utero* antigen-exposed lambs in response to the infection. The rapid migration of these lymphocytes to the target site of infection, the gut, is indicative of an anamnestic response. It is possible that cells within the IPP could have been modulated by the *in utero* antigen-exposure. They seem to have

retained memory of the antigen as well and on re-exposure to the antigen in postnatal life seem to be responding in an anamnestic fashion. This rapid depletion of TNF-α^+ lymphocytes from the IPP of *in utero* antigen-exposed lambs was not effective in reducing worm count in the gut.

Among control lambs, when the TNF-α levels in the jejunum and MLN were low the corresponding level in the IPP was high. The mucosa of the IPP from the control lambs contained a large number of TNF-α^+ lymphocytes. Thus it appears that a large number of these TNF-α^+ lymphocytes were still retained in the IPP mucosa even with the ongoing infection in the jejunum. The retention of TNF-α^+ lymphocytes within the IPP of control lambs may have been beneficial to the animals because they had fewer clinical symptoms and a marginally lower worm burden in the gut.

INTERLEUKIN-1β

INTRODUCTION

The interleukin-1 (IL-1) family comprises two important members viz. IL-1 α and IL-1 β. They are biochemically distinct polypeptides produced by activated mononuclear cells and other cell types. Both subtypes of murine and human IL-1 are initially synthesised as a 31 KDa pro IL-1 molecule (Gray *et al.*, 1986; March *et al.*, 1985). Pro IL-1 α has biological activity and remains predominantly cell-associated. However, pro IL-1 β is inactive until it is enzymatically cleaved and released as an active molecule. A specific enzyme, interleukin I β converting enzyme, is responsible for this (Cerretti *et al.*, 1992). IL-1 β binds to two different receptors, namely type I, which is expressed on T cells and fibroblasts, and type II, which is expressed on B cells, bone marrow, monocytes and polymophonuclear cells (Dinarello, 1991). Despite the significant differences in amino acid sequence between IL-1 α and IL-1 β, both forms share most of their biological activities and share the same cell surface receptors (Dinarello, 1991).

Many biological activities have been attributed to IL-1 β (Dinarello, 1991). The expression of IL-1 β in tissues is generally regarded as a proinflammatory response. Thus, elevated IL-1 β levels are commonly encountered in inflammatory bowel disease (Woywodt *et al.*, 1999). The IL-1 β released at the site of inflammation activates T lymphocytes, endothelial cells and fibroblasts to express cell surface markers and release further cytokines like TNF α, IL-6 and IL-8 (Bachert *et al.*, 1998). There is a dramatic increase in IL-1 production by a variety of cells in response to infection, microbial toxins, inflammatory agents and products of activated lymphocytes (Dinarello, 1991).

There have been a few reports of the importance of IL-1 β expression in sheep tissue. Rothel *et al.* (1997) could not detect IL-1 β positive cells in the normal sheep lymph nodes although macrophages could be readily demonstrated by immunohistochemistry. In contrast, the lymph nodes from *Corynebacterium pseudotuberculosis*-infected animals had IL-1 β positive cells which were localized in a layer of macrophages surrounding the necrotic tissue.

Following the subcutaneous inoculation of sheep with either the virulent or the vaccinal strain of *Salmonella abortus ovis*, the frequency of detection of IL-1 β in the efferent lymph cells of the prescapular lymph node was increased (Gohin *et al.*, 1997).

In sheep naturally infected with paratuberculosis the infected ileal tissue expressed a significantly increased level of mRNA for IL-1 β in comparison with control animals (Alzuherri *et al.*, 1996).

Although IL-1 β has a well established image as a proinflammatory cytokine in clinical conditions such as inflammatory bowel disease and HIV (Arai *et al.*, 1998; Bode *et al.*, 1998) it has been shown to have other vital biological properties *in vivo*.

Growth promoting effects of IL-1 β: IL-1 has potent hematopoietic effects. Hematopoiesis may be affected at various levels. IL-1 induces the production of GM-CSF, G-CSF, M-CSF, IL-3 and other cytokines. It also regulates the cell cycle of progenitor cells. IL-1 by itself has no effect on stem cell proliferation or differentiation but acts on hematopoiesis synergistically with CSFs and other cytokines (Dinarello, 1991; Dinarello, 1992).

The investigations of Pezzano *et al.* (1996) have demonstrated that maturation of thymocytes within the thymic nurse cells requires the costimulatory effects of IL-1 β. IL-1 β has been shown to enhance the proliferative response of human B cells. It stimulates the proliferation of splenic B cells, both by crosslinking of their surface immunoglobulin (Freedman *et al.*, 1988) and by means of signals through CD40 and IL-4R (Rousset *et al.*, 1991).

A variety of cytokines appear to influence the development of germinal centres. Germinal centres represent foci of rapid B cell proliferation and differentiation in secondary lymphoid tissue. They consist of B cells and a dense network of follicular dendritic cells, germinal centre T lymphocytes and macrophages (Butch *et al.*, 1993; Toellner *et al.*, 1995). The follicular dendritic cells appear to influence the survival of germinal centre B cells and may produce cytokines important for germinal centre B cell proliferation (Butch *et al.*, 1993). The process of B cell development in the germinal centres can be divided into proliferation, somatic mutation and selection. The germinal centre B cells exhibit a high dependence on the surrounding stromal support for their growth which

requires both direct cell contact and the presence of cytokines (Pound & Gordon, 1997).

Pound and Gordon (1997) investigated the ability of cytokines, used individually and in combination, to stimulate long term DNA synthesis and growth of germinal centre B cells *in vitro*. The triple combination of interleukins IL-10, IL-2 and IL-1 β could maintain high levels of germinal centre B cell stimulation for at least 10 days.

Cytokine gene expression by the germinal centres of human tonsils was investigated by Toellner *et al.*(1995). They observed that a small population of follicular dendritic cells produced high levels of IL-1 β mRNA. They were also able to demonstrate the actual synthesis of IL-1 β protein using immunohistochemistry. IL-1 β can also be produced in large amounts by follicular dendritic cells in certain diseases like AIDS (Gerdes *et al.*, 1990).

Some investigators have failed to demonstrate a significant role for IL-1 β in the development of germinal centres. Butch *et al.* (1993) observed that the germinal centre B and T cells of human tonsils were consistently negative for expression of IL-1 β mRNA. Non-germinal centre T cells from a few samples were occasionally positive for IL-1 β.

Role of IL-1 β in intestinal maturation: There are suggestions that IL-1 β might be involved in the development and maturation of the gut. IL-1 is able to regulate sucrase-isomaltase gene expression, a marker of gut differentiation , in the human intestinal cell line Caco-2 (Molmenti *et al.*, 1993).

Mengheri *et al.* (1996) investigated whether the expression of cytokines in the intestine of rats alters at the time of weaning. An interesting finding which they reported was that IL-1 β was the only cytokine to show differing levels of expression at different ages. In the Peyer's patch, the level of IL-1 β expression was very low at 16 days of age. Following this, the levels increased by 19 days and then peaked at 21 days of age a time at which the intestinal epithelium passes through a major stage of differentiation. At 30 days of age there was a marginal decrease in the level of IL-1 β detected. Mengheri *et al.* (1996) have postulated that IL-1 β is an important participant in the physiological processes that takes place in the intestine at the time of weaning. They believe that IL-1 β could take

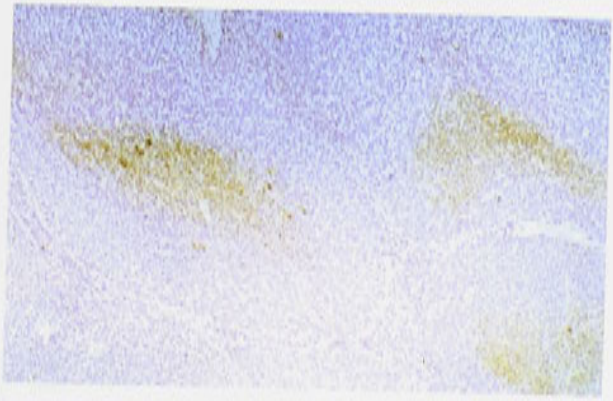
Table- 10.1 :Total positive area – IL-1 β

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	61 ± 44	290 ± 198	NS
Mesenteric lymph node *(cortex)*	2075 ± 381	2906 ± 572	NS
Mesenteric lymph node *(Medulla)*	719 ± 307	717 ± 238	NS
Ileal Peyer's patch *(Mucosa)*	542 ± 254	1119 ± 272	P=.06
Ileal Peyer's patch *(Follicle)*	25662 ± 7206	51638 ± 10179	P=.023

Fig-L: Follicles of the MLN staining for IL-1 β. The lymphocytes within the follicles are stained with a low intensity. Darker staining cells with dendritic cell morphology can be seen dispersed within some follicles. This pattern of staining for IL-1 β within the MLN was much more evident among control lambs in comparison with *in utero* antigen-exposed lambs.

Fig-M: Follicles of the IPP staining for IL-1 β. The positive cells are confined to an inner core leaving a thin peripheral zone of unstained cells. Dispersed between the lightly stained lymphocytes are darker staining bodies with dendritic cell morphology.

Fig-L:



80 μm

Fig-M:

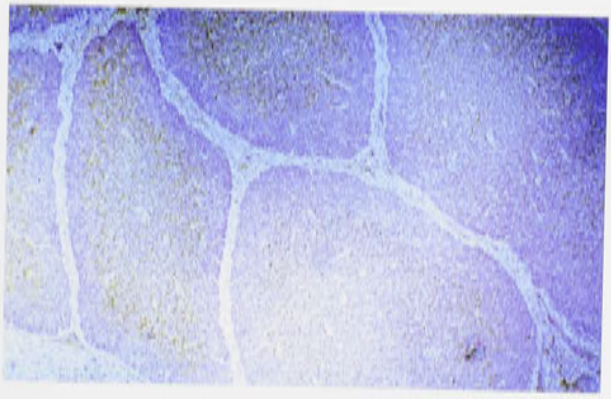


Fig-10.2 : Frequency of detection of IL-1 β in the jejunum

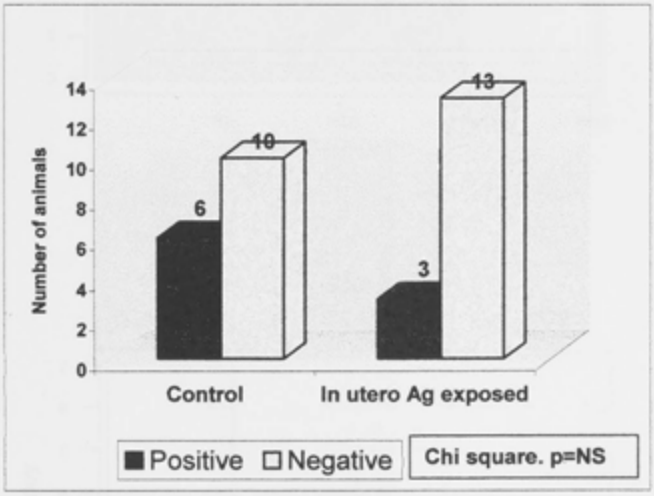


Fig-10.3 : Frequency distribution of IL-1 β in the cortex of the MLN

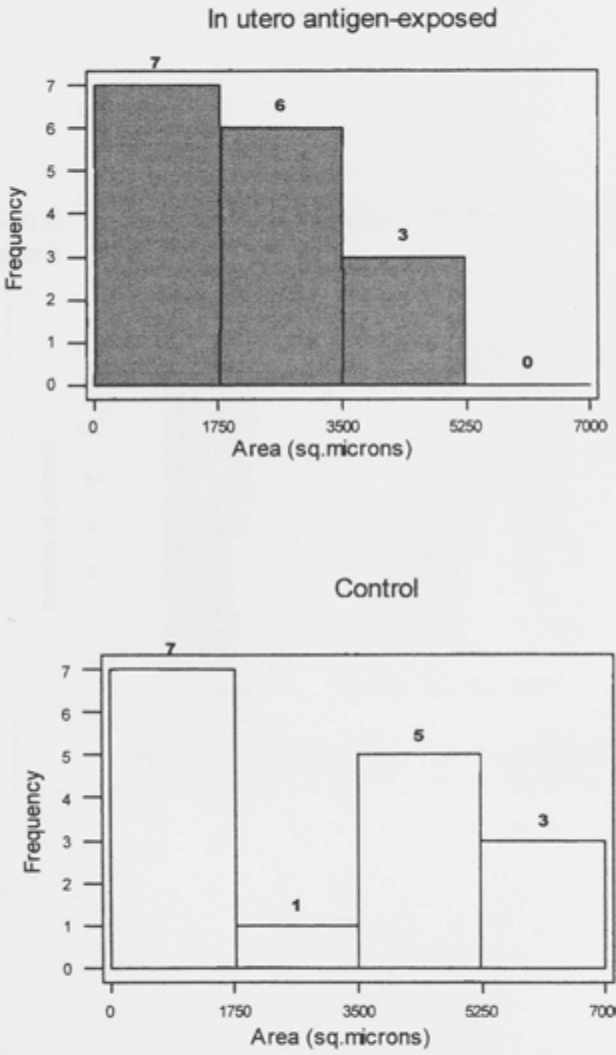


Fig-10.4 : Frequency of detection of IL-1 β in the IPP mucosa

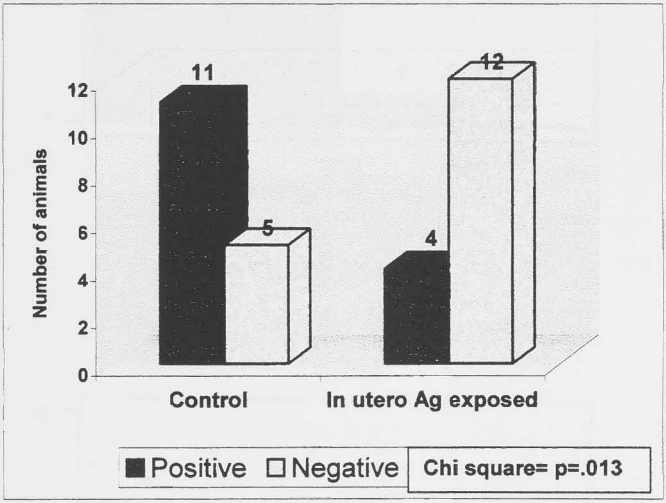
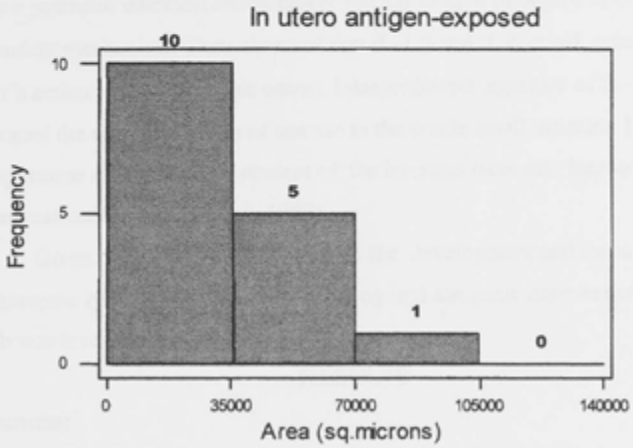
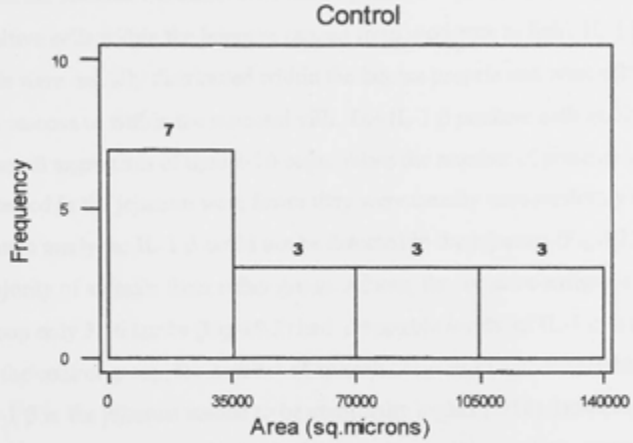


Fig-10.5 : Frequency distribution of IL-1 β within the IPP follicles





part in the regulation or activation of epithelial differentiation. Likewise, IL-1 β may also be involved in the structural and functional changes that occur within the Peyer's patch tissue.

The work of Kaouass *et al.*(1997) suggests that dietary polyamines could induce postnatal intestinal development and maturation through a cytokine-dependent mechanism. They observed that IL-1 β and IL-6 could mimic each other's action to a considerable extent. Intraperitoneal injection of IL-1 β increased the specific activity of sucrase in the whole small intestine. Likewise, the spermine and spermidine content of the intestine were also increased by the same treatment (Kaouass *et al.*, 1997).

Given the importance of IL-1 β in the development and maturation of the gut immune system its expression following oral antigenic exposure of the foetal lamb was investigated.

RESULTS

Jejunum:

Histology: Histological differences were not evident between the *in utero* antigen-exposed and the control group of animals. Most of the IL-1 β positive cells had morphological features typical of lymphocytes. Rarely, larger cells with dendritic features were also seen staining for IL-1 β. The intensity of staining of positive cells within the jejunum ranged from moderate to light. IL-1 β positive cells were usually distributed within the lamina propria and were seldom seen in the mucosa or within the mucosal villi. The IL-1 β positive cells could be found in small aggregates of upto 4-10 cells. When the number of positive cells detected in the jejunum were fewer they were usually seen randomly distributed.

Image analysis: IL-1 β could not be detected in the jejunum (Fig-10.2) in the majority of animals from either group. Among the *in utero* antigen-exposed group only 3/16 lambs (Fig-10.2) had detectable levels of IL-1 β in the jejunum. In the control group, the number of animals (Fig-10.2) with detectable levels of IL-1 β in the jejunum tended to be marginally higher (6/16). However, the difference in numbers of animals, between the *in utero* antigen-exposed and control groups, was not statistically significant by chi square analysis.

Although not statistically significant, the mean area staining for IL-1 β in the jejunum of control lambs was greater in comparison with those in the *in utero* antigen-exposed lambs (Table-10.1).

Mesenteric Lymph node (Cortex):

Histology: The average size of follicles staining for IL-1 β tended to be higher in the control group of animals in comparison with the *in utero* antigen-exposed group of lambs. Within the cortical region of the MLN, monoclonal antibody to IL-1 β specifically stained follicular cells. The antibody stained none of the lymphocytes found in the interfollicular region. The positive cells within the follicles were stained with a light intensity. Follicular dendritic cells were sometimes observed to be stained within these follicles. These follicular dendritic cells had irregular dendritic morphology and tended to stain more intensely. In those instances in which positive follicular dendritic cells were detected in the follicles, the overall intensity of staining of the follicle was considerably higher in comparison with other follicles in which follicular dendritic cells were not positively stained. On high power examination of the IL-1 β^{+} follicles, the supporting stromal tissue was also observed to be stained lightly.

Image analysis: In contrast to the jejunum, the majority of animals in both groups had IL-1 β detectable by immunohistochemistry in the cortical region of the MLN. Among the *in utero* antigen-exposed group, 15/16 lambs had IL-1 β which were detectable by immunohistochemistry. In the control group of animals the numbers were marginally lower with 13/16 animals having IL-1 β positive cells within the cortical region of the MLN.

The mean area staining for IL-1 β in the cortical region of the MLN was considerably higher in the control group compared with the *in utero* antigen-exposed group of lambs (Table-10.1). The mean positive area detected in the control lambs was 28.5% greater than that observed in the *in utero* antigen-exposed group although the difference between the two groups was not statistically significant.

When the area stained by IL-1 β within the cortex of the MLN was divided into four equal ranges and the frequency distribution calculated, distinct differences between the two groups were apparent. The majority of animals from

the *in utero* antigen-exposed group (13/16) had a positive area <3500 sq.μ (Fig-10.3). In the control group only 8/16 animals had a small area (<3500 sq.μ) positive for IL-1 β (Fig-10.3). There were only three animals from the *in utero* antigen-exposed group with large area (>3500 sq.μ) of IL-1 β (Fig-10.3). In contrast, 50% of animals from the control group had large area (>3500 sq.μ) of IL-1 β in the cortex of the MLN (Fig-10.3).

Mesenteric lymph node (Medulla):

Histology: Most of the positive cells were lymphocytes. Occasionally a few larger cells with dendritic morphology were also positive for IL-1 β. The intensity of staining of positive cells ranged from medium to low. In those instances in which a larger area was stained, the cells were found in small to medium sized clusters between the trabeculae. When the area staining for IL-1 β was less, the cells were usually found randomly distributed within the medullary region.

Image analysis: There was no difference in the mean area staining for IL-1 β in the medulla of the MLN in the two groups of lambs (Table-10.1). The majority of animals from both the *in utero* antigen-exposed and control groups had IL-1 β detectable by immunohistochemistry, in the medulla of the MLN. There were 12/16 lambs in the *in utero* antigen-exposed group with IL-1 β^+ cells in the medulla while in the control group there were 13/16 animals.

Ileal Peyer's Patch (Mucosa):

Histology: The majority of IL-1 β positive cells were clustered within the mucosal villi. Occasionally, cells were also seen randomly distributed within the interfollicular region. The IL-1 β positive cells were invariably lymphocytes. Positive cells with dendritic morphology were only rarely seen. The intensity of staining of the positive cells ranged from medium to high. In the *in utero* antigen-exposed group the number of IL-1 β positive cells was considerably less than in the control group of animals.

Image analysis: The mucosa of the IPP from the majority (12/16) of *in utero* antigen-exposed lambs did not contain IL-1 β^+ cells (Fig-10.4). In contrast, among the control group, 11/16 animals had IL-1 β^+ cells within the mucosa of the IPP (Fig-10.4). This difference in the number of animals in the *in utero*

antigen-exposed and control groups of animals with detectable area of IL-1 β in the mucosa of the IPP was significant (p=.013).

The mean area staining for IL-1 β was considerably higher (p=.06) in the control group in comparison with the *in utero* antigen-exposed group of lambs (Table-10.1).

Ileal Peyer's Patch (Follicle):

Histology: Staining for IL-1 β within the follicles of the IPP was confined to the inner zone leaving a thin periphery of unstained lymphocytes. The cells from the corona of the follicle were also not stained. Positive lymphocytes were stained with low intensity. Within the follicles, follicular dendritic cells were also occasionally stained for IL-1 β. These follicular dendritic cells were irregularly shaped with cytoplasmic projections arising from their cell membrane. When the follicular dendritic cells were stained for IL-1 β, the surrounding lymphocytes also stained with greater intensity. IL-1 β was also detected in the extracellular spaces of the lymphocytes and the supporting stroma.

Image analysis: Among both groups of lambs, IL-1 β could be detected within the IPP follicles in the majority of animals. Of the *in utero* antigen-exposed lambs 11/16 animals had detectable area of IL-1 β within the follicles of the IPP. Among the control group 14/16 animals had IL-1 β positive cells within the follicles.

The mean area staining for IL-1 β within the follicles of the IPP was significantly greater (p=.023) in the control group of animals in comparison with the *in utero* antigen-exposed group of lambs (Table-10.1).

The frequency distribution of the positive area also revealed differences between the two groups (Fig-10.5). In the *in utero* antigen-exposed group of animals there was only one animal (Fig-10.5) with a positive area >70,000 sq.μ. In contrast, among the control group of lambs there were 6/16 animals (Fig-10.5) with a positive area >70,000 sq.μ.

DISCUSSION

The B lymphocytes and the surrounding stromal tissue of the follicles stained with a light intensity for IL-1 β. Occasionally, follicular dendritic cells staining with high intensity were also seen within some IL-1 β positive follicles.

In the case of those animals in which IL-1 β dendritic cells could be detected in the follicles, the overall intensity of staining of the follicles was considerably higher in comparison with other follicles in which follicular dendritic cells were not positively stained. The results of the present study are identical with those of Toellner *et al.* (1995) who investigated cytokine gene expression by the germinal centres of human tonsils. They observed that a small population of follicular dendritic cells produced high levels of IL-1 β mRNA. In addition to the mRNA, they were also able to demonstrate the presence of the specific translated proteins using immunohistochemistry. IL-1 β can also be produced by follicular dendritic cells in high amounts in certain diseases like AIDS (Gerdes *et al.*, 1990). It appeared that IL-1 β produced by the follicular dendritic cells was diffusing into the surrounding follicular tissue. This was supported by the observation in the present experiments that when the follicular dendritic cells were stained the intensity of staining of the surrounding tissue was also correspondingly higher. The follicular dendritic cells could be initially synthesizing the inactive pro-IL-1 β form of the molecule which is subsequently cleaved and released into the surrounding tissue by the IL-1 β converting enzyme. The active form of IL-1 β can bind to the type II receptors expressed on B cells (Dinarello, 1991). It is also possible that B lymphocytes of the follicles might be synthesizing this protein at very low levels. It was not possible to make a clear distinction between these two possibilities in the present study.

The *in utero* antigenic exposure of foetal lambs to *Trichostrongylus* larval antigen brought about substantial changes in the expression of IL-1 β within the B cell follicles of the IPP and the cortical region of the MLN. The mean area staining for IL-1 β within the follicles of the IPP was significantly greater in the control group of animals in comparison with the *in utero* antigen-exposed lambs. Likewise, the mean area staining for IL-1 β within follicles of the MLN cortex was 28.5% greater than that observed in the *in utero* antigen-exposed group of lambs. Thus it appears that the *in utero* antigenic exposure followed by re-exposure to the homologous antigen in postnatal life could markedly curtail the expression of IL-1 β within B cell follicles of the IPP and MLN.

Cytokines are thought to play a critical role in the development and maturation of B cell follicles. Pound and Gordon (1997) investigated the ability of cytokines, used individually and in combination, to stimulate long term synthesis and growth of germinal centre B cells *in vitro*. They observed that the triple combination of interleukins IL-10, IL-2 and IL-1 β could maintain high levels of germinal B cell stimulation for at least 10 days. IL-1 β has been shown to stimulate the proliferation of splenic B cells both by crosslinking of their surface immunoglobulins (Freedman *et al*., 1988) and by signaling through CD40 and IL-4R (Rousset *et al*., 1991). Taking the published evidence into account it is possible that the curtailed expression of IL-1 β among *in utero* antigen-exposed lambs could have adversely affected the development and maturation of the B cell follicles in the IPP and MLN.

The *in utero* antigenic exposure also brought about significant changes in the expression of IL-1 β within the mucosa of the IPP. The mucosa of the IPP from the majority of *in utero* antigen-exposed lambs did not contain IL-1 β positive lymphocytes. In direct contrast, among the control group the majority of lambs had IL-1 β positive cells within the mucosa of the IPP. The mean area staining for IL-1 β within the mucosa of the IPP was significantly greater in the control group in comparison with the *in utero* antigen-exposed lambs. Thus it appears that *in utero* antigenic exposure followed by re-exposure to the homologous antigen can significantly curtail the expression of IL-1 β positive lymphocytes within the IPP mucosa. It appears that a large numbers of these positive lymphocytes were T lymphocytes since large numbers of IL-1 β-positive cell were present even when very few B lymphocytes ($CD45R^+$ lymphocytes) could be detected within the mucosa of the IPP. The IL-1 β positive cells originating within the IPP, could migrate into other effector sites like the jejunum to initiate a proinflammatory immune response.

IL-1 β could not be detected in the jejunum of the majority of animals from either group. However, among control lambs the number of animals with detectable area of IL-1 β tended to be marginally higher in comparison with *in utero* antigen-exposed animals. The failure of young lambs to express IL-1 β within the jejunum could be one of the factors contributing to their increased susceptibility to *Trichostrongylus colubriformis* infection.

A consistent observation from the present study was that *in utero* oral antigenic exposure markedly curtailed the expression of IL-1 β within the gut tissues. There is evidence to suggest that IL-1 β might be involved in the development and maturation of the gut. Mengheri *et al.* (1996) have shown that IL-1 β is the only cytokine to show different levels of expression within the gut of rats at different ages. They observed that young animals had very low levels of IL-1 β expression in the gut, which increased as the animals became older. The work of Kaouass *et al.* (1997) suggested that dietary polyamines could induce postnatal intestinal development and maturation though an IL-1 β dependent mechanism. Likewise, they also observed that the intraperitoneal injection of IL-1 β increased the expression of maturation markers within the gut tissue. There is a possibility that, among the *in utero* antigen-exposed lambs, the reduced expression of IL-1 β could have interfered with the development and maturation of the gut.

INTERLEUKIN-2

INTRODUCTION

Interleukin-2 (IL-2), originally called T cell growth factor, is an important cytokine produced by $CD4^+$ T cells, and in lesser quantities by $CD8^+$ T cells (Fitch *et al.*, 1993). $CD4^+$ T cells particularly of the Th0 and Th1 subsets secrete large amounts of this cytokine (Mosmann & Sad, 1996). IL-2 exerts an effect on the same cells that produce it (i.e., functions as an autocrine growth factor). IL-2 acts on nearby T lymphocytes, including $CD4^+$ and $CD8^+$ cells, and is therefore also a paracrine growth factor. It promotes T cell proliferation, differentiation of B cells and activates macrophages, natural killer cells and lymphokine-activated killer cells (Smith, 1992).

The conditions following initial stimulation of T cells *in vivo* influence the nature of the effector cells generated and the types of lymphokines produced on restimulation. The presence of IL-2 results in a Th1 response characterised by cells secreting large amounts of IL-2 and interferon γ. Although IL-2 generally favours a polarised Th1 response it has been shown also to be necessary for the maturation of a Th2 type response (Ben-Sasson *et al.*, 1990). The effects of IL-2 are mediated through a specific cell surface receptor named IL-2R or CD25. The interaction of IL-2 with the CD25 receptor promotes proliferation of the antigen specific T cells, thus amplifying the specific immune response. In addition, IL-2 may also deliver a signal for differentiation to specific lymphocytes or in some cases, inhibit lymphocyte growth. Thus the synthesis of IL-2 and expression of IL-2R by T cells play a central role in both the nature and magnitude of the immune response (Zeitz *et al.*, 1988).

IL-2 expression in the gastrointestinal tract: Within the gut of foetal mice, IL-2 has been first detected in association with a few discrete, isolated cells at 16 days of gestation. As gestation progresses the number of IL-2 reactive cells increases in frequency (Yang-Snyder & Rothenberg, 1998). Murray *et al.* (1998), in contrast, were not able to demonstrate the expression of IL-2 genes in the murine foetal intestine.

Cytokine gene expression in the intestine of perinatal rats at the time of weaning has been investigated by Mengheri *et al.* (1996). No variation in the level of expression of IL-2 mRNA was detected during this period. Based on this

observation they have suggested that IL-2 might not play a significant role in the intestinal maturation process of perinatal rats. IL-2 gene knockout mice also develop normally during the first 3-4 weeks of postnatal life supporting the observation that IL-2 might play only an insignificant role in intestinal maturation (Sadlack *et al.*, 1995). However, at a later stage of life these IL-2 knockout mice become severely immunocompromised and about 50 % of the animals die between 4-9 weeks after birth. Of the remaining mice, 100 % develop an inflammatory bowel disease with striking clinical and histological similarity to ulcerative colitis in humans. The abnormalities observed in the immune system included a high number of activated T and B cells, elevated immunoglobulin secretion, anti-colon antibodies, and aberrant expression of class II MHC molecules (McDonald *et al.*, 1997; Sadlack *et al.*, 1993). An uncontrolled activation and proliferation of $CD4^+$ T cells can trigger a generalised autoimmune disease in IL-2 deficient mice (Sadlack *et al.*, 1995).

It is not clear why the deficiency of IL-2 should result in hypersensitivity to a variety of self and non-self antigens. It has been proposed that IL-2 is required to maintain self tolerance and perhaps, in the case of gut, tolerance to the flora as well (MacDonald, 1995). The relevance of tolerance to gut flora has been questioned recently by Schultz *et al.* (1999) who demonstrated that IL-2-deficient mice raised under germ free conditions still developed delayed, mild focal intestinal inflammation. There seems to be considerable difference across animal species in the leukocyte population of the gut in terms of IL-2 expression capability as well as response to exogenous IL-2.

Unstimulated lymphocytes derived from the mesenteric lymph node and lamina propria of normal non-human primates do not synthesise IL-2 in culture. However, lamina propria lymphocytes produce significantly more IL-2 after mitogen stimulation than lymphocytes from the peripheral blood or spleen. Although the levels of IL-2 induced in MLN lymphocytes after mitogen stimulation was low it was considerably higher than that induced in lymphocytes from the peripheral blood and spleen (Zeitz *et al.*, 1988). Zeitz *et al.* (1988) also observed that lamina propria lymphocytes underwent a significantly higher rate of proliferation in response to exogenous IL-2 compared with lymphocytes from the blood, spleen and MLN. The peculiar feature of lamina propria lymphocytes

of primates is that very little IL-2 expression is observed in the unstimulated cells whereas a significant number of these lymphocytes express the IL-2 receptor CD25, a marker for T cell activation. Although their numbers are smaller compared to lamina propria cells, lymphocytes isolated from the MLN also contain substantial numbers of CD25 positive cells in comparison with lymphocytes from the spleen and blood.

In the case of neonatal piglets (15 to 47 days old), both the intraepithelial leucocytes and the MLN lymphocytes were identical in that they produced very little IL-2 in response to conA mitogen stimulation (Whary *et al.*, 1995). Lamina propria lymphocytes of pigs stimulated with mitogen underwent an initial burst of IL-2 transcription but within a few hours it was shut down. By 12 hours, the cytokine profile of these cells had become highly polarised with high levels of IL-4 transcripts and an absence of IL-2 mRNA (Bailey *et al.*, 1998). Bailey *et al.* (1998) have suggested that the microenvironment in the porcine gut favours the differentiation of Th2 type effector cells which are capable of local antigen recognition without the release of tissue-damaging cytokines. The intraepithelial leukocytes from piglets (15 to 47 days old) exhibited a high proliferative response to exogenous IL-2. Unlike the intraepithelial leukocytes, MLN lymphocytes were unresponsive to exogenous IL-2 (Whary *et al.*, 1995). The reduced response of MLN lymphocytes to IL-2 indicates that IL-2 receptor expression within these cells is low. This has led Whary *et al.* (1995) to speculate that MLN lymphocytes are in a lower state of activation compared to intraepithelial leukocytes.

In the case of Merino sheep, Premier and Meeusen (1998) did not observe any difference in the amount of IL-2 mRNA induced in the cells obtained from peripheral or gastrointestinal lymph nodes after polyclonal stimulation. Based on this observation Premier and Meeusen (1998) suggested that cells capable of expressing Th1 cytokines are present in both types of lymph nodes.

There is considerable variation in the extent of expression of IL-2 within the gut of infected animals. Svetic *et al.* (1993) investigated the IL-2 response in the MLN and Peyer's patches of mice, at various time points after infection with the nematode parasite, *Heligmosomoides polygyrus*. They observed that IL-2 cytokine expression showed no changes during the course of infection. Although

IL-2 was not elevated in either the MLN or Peyer's patches, a marked increase in other cytokines like IL-4, IL-5 and IL-9 was observed.

Schito *et al.* (1998) infected mice with the protozoan parasite *Eimeria papillata* to characterise lymphocyte responses and cytokine profile through the course of primary and secondary infection. Lymphocytes from the MLN had an elevated expression of IL-2 during the secondary infection. Additionally, during the secondary infection IL-2 and interferon γ were detected earlier (Days 1-2) when compared to the primary infection (Days 5-20). Schito *et al.* (1998) have suggested that the rapid and increased IL-2 response might play a central role in maintaining or stimulating effector memory cells during the secondary infection.

Almeria *et al.* (1997) quantified the cytokine gene expression in lamina propria lymphocytes of cattle infected with the gastrointestinal nematode *Ostertagia ostertagi.* They observed that the lamina propria lymphocytes expressed very low levels of IL-2. Furthermore, there was no difference between the infected and control animals in the amount of IL-2 induced. Canals *et al.* (1997) were another group to investigate the cytokine profile induced in the abomasal lymph node of calves after a primary infection with *Ostertagia ostertagi.* They observed that the lymphocytes from two of the three uninfected animals expressed high levels of IL-2 mRNA after mitogen stimulation. However, in infected calves, mRNA levels for IL-2 decreased as early as four days post-infection and remained low through to day 21 before returning to preinfection levels 28 days after infection. Analysis of the unstimulated abomasal lymphocytes from infected animals also revealed that IL-2 mRNA levels decreased five fold by 11 days after infection. The IL-2 levels remained depressed until the 28th day post infection. Canals *et al.* (1997) believes that the diminished capacity of the abomasal lymph node to respond to mitogenic stimulation may be the result of a generalised decrease in T lymphocyte reactivity. Alternatively it may also indicate a down-regulation of the Th1 type responses (Canals *et al.*, 1997).

Among sheep clinically infected with *Myocbacterium avium* ssp *paratuberculosis* the lesions observed within the gut can be categorised into two namely the tuberculoid (paucibacillary) form of the lesion and the lepromatous (multibacillary) form. The expression of cytokines in the gut of paratuberculosis

Table- 11.1 : Total positive area – IL-2$^+$ cells

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	292 ± 92	318 ± 112	NS
Mesenteric lymph node	301 ± 137	633 ± 212	NS
Ileal Peyer's patch	1305 ± 364	1263 ± 266	NS

Fig-11.2: Frequency of occurrence of IL-2$^+$ cells in the jejunum

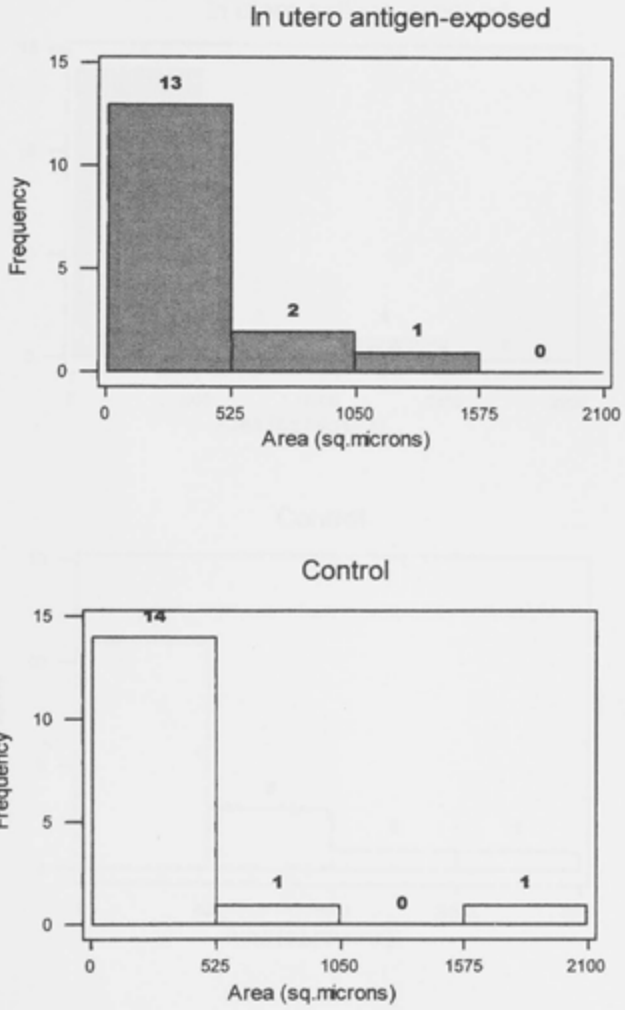


Fig-11.3: Frequency of occurrence of IL-2$^+$ cells in the MLN

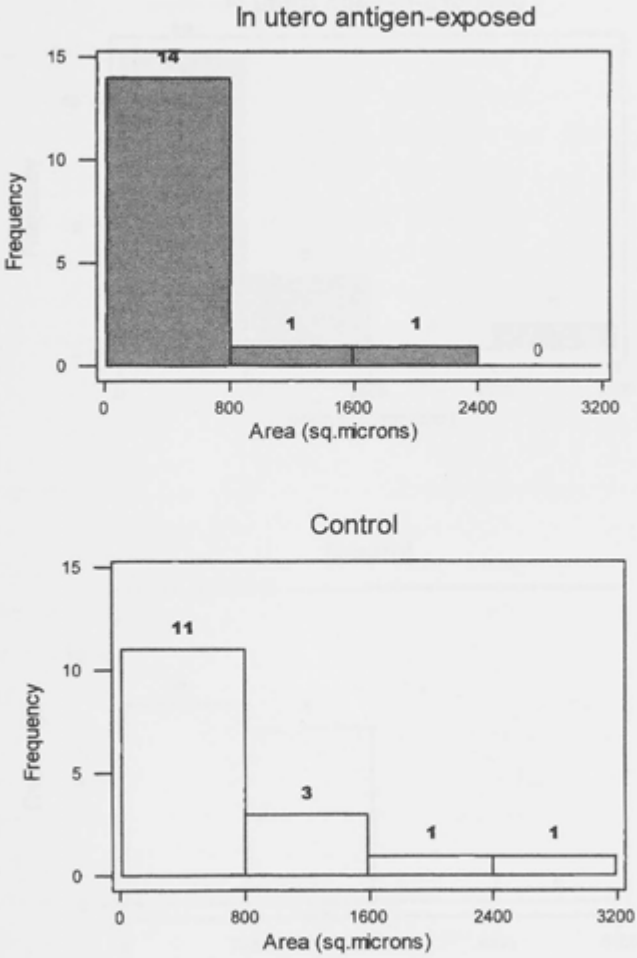
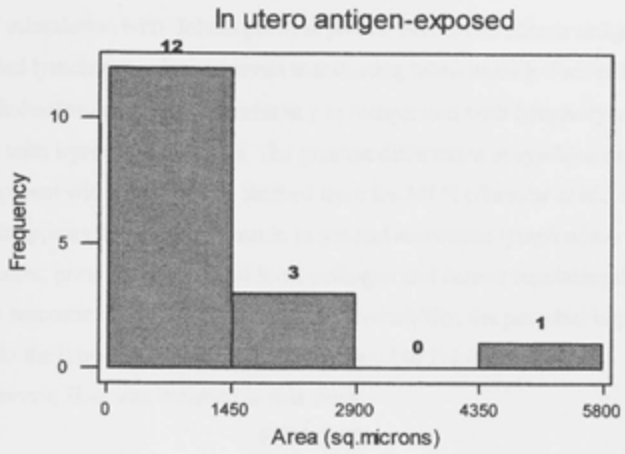
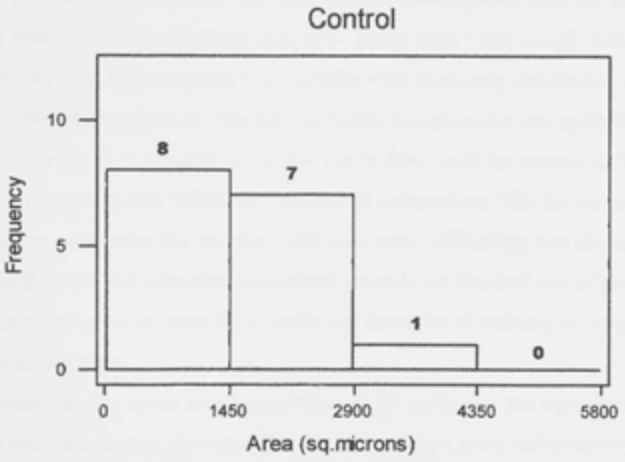


Fig-11.4: Frequency of occurrence of IL-2$^+$ cells in the IPP





(Johne's disease) infected sheep has been shown to be related to the type of lesions present. Burrells *et al.* (1999) investigated the capacity of lymphocytes from blood, MLN and ileal lamina propria to secrete IL-2 and interferon γ after *in vitro* stimulation with Johnin purified protein derivative. Johnin antigen-stimulated lymphocytes from animals manifesting tuberculoid lesions exhibited greater induction of IL-2 and interferon γ in comparison with lymphocytes from animals with lepromatous lesions. The greatest differences in cytokine expression were apparent with lymphocytes derived from the MLN (Burrells *et al.*, 1999).

It appears that IL-2 expression in gut and associated lymph nodes varies with species, presence and type of local pathogen and factors regulating the gut immune response to the infection. Given this variability, the potential importance of IL-2 to the type of immune response generated to *Trichostrongylus colubriformis*, IL-2 was included in this study.

RESULTS

Jejunum

Histology: Few histological differences were apparent between jejunal tissues from the two groups of animals. The majority of animals, from both the groups, had very few IL-2$^+$ cells within the jejunum. These cells were usually stained with low intensity. Positive cells were usually seen randomly distributed within cross sections of the jejunum. The intraepithelial lymphocytes and epithelium were negative for IL-2 expression. A few lambs from both the groups had larger numbers of positive cells within the jejunum in comparison with the remainder of the lambs. In such cases the positive cells were seen infiltrating into the lamina propria of the villi and sometimes clustered towards the luminal end of the villi. When there tended to be more IL-2$^+$ cells, the intensity of staining of the positive cells was also higher.

Image analysis: The mean area occupied by IL-2$^+$ cells was not significantly different between the two groups of lambs. There was a marginal tendency for the area stained to be higher (8 %) in the control group of lambs in comparison with the *in utero* antigen-exposed lambs (Table-11.1).

The frequency distribution of animals in relation to the area stained by IL-2$^+$ cells in the jejunum was not appreciably different between the two groups (Fig-11.2)

Mesenteric Lymph Node

Histology: The majority of *in utero* antigen-exposed lambs had fewer IL-2$^+$ cells within the MLN. These cells tended to be lightly stained and were randomly distributed within the tissue as single cells or small clusters (4-8 cells). In contrast, control lambs had larger numbers of IL-2$^+$ cells within the MLN. The intensity of staining did not differ significantly from that of the *in utero* antigen-exposed lambs, with positive cells staining with low intensity. The positive cells were usually found in small (4-8 cells) to medium (10-15 cells) clusters in the control group of lambs.

Image analysis: Although the difference was not statistically significant, the mean area staining for IL-2$^+$ cells was substantially higher in the control group of lambs in comparison with the *in utero* antigen-exposed lambs. The mean level of IL-2$^+$ area was 52.5 % higher in the control group in comparison with the *in utero* antigen-exposed lambs (Table-11.1).

The frequency distribution of animals in relation to the area stained by IL-2$^+$ cells in the MLN revealed some marginal differences between the two groups. The number of animals with an IL-2$^+$ area <800 sq.μ (Fig-11.3) was marginally higher (14/16) in the *in utero* antigen-exposed lambs in comparison with the control lambs (11/16). There were only two lambs (Fig-11.3) with an IL-2$^+$ area >800 sq.μ while in the control group there were five lambs with the same IL-2$^+$ area (>800 sq.μ).

Ileal Peyer's patch:

Histology: Histological differences were not evident between the *in utero* antigen-exposed and control groups of lambs. The majority of IL-2$^+$ cells were confined to the lamina propria of the mucosal villi. When the number of positive cells tended to be high within the villi they were observed to be clustered towards the luminal end of the villi. A few positive cells were also seen within the periphery of the interfollicular region. On rare occasions a few large, irregularly shaped cells within the follicles stained for IL-2. The follicle including the dome region was consistently negative for IL-2 expression. The IL-2$^+$ cells within the IPP stained with moderate intensity.

Image analysis: The mean area staining for IL-2 was almost identical in the two groups of lambs (Table-11.1).

The frequency distribution of animals in relation to the area occupied by IL-2$^+$ cell in the IPP revealed some appreciable differences between the two groups. Among the *in utero* antigen-exposed lambs 75 % of the animals (Fig-11.4) had an IL-2$^+$ area <1450 sq.μ while in the control group only 50 % of the animals had the same positive area (<1450 sq.μ). There were fewer animals (4/16) in the *in utero* antigen-exposed group (Fig-11.4) with an IL-2$^+$ area >1450 sq.μ, while in the control group there were 8/16 lambs with the same positive area (>1450 sq.μ).

DISCUSSION

IL-2 is a key regulator of immune and inflammatory responses. It promotes T cell proliferation and differentiation of B cells and activates macrophages, natural killer cells and lymphokine-activated killer cells (Smith, 1992). In the present study the MLN was the only lymphoid tissue to manifest an appreciable difference in IL-2 expression when the two groups of lambs were compared. The mean area staining for IL-2 was substantially greater (although the difference was not significant statistically) in the MLN of the control group of animals in comparison with the *in utero* antigen-exposed lambs. Lambs which were exposed to the soluble, third stage larval antigen of *Trichostrongylus colubriformis in utero,* mounted a very poor IL-2 response in the MLN on re-exposure to the live larvae in post natal life.

The IL-2 response in the MLN to an antigenic stimulus can be down-regulated if the animal is initially sensitised with the same antigen by the oral route. The MLN cells of mice tolerised by the oral feeding of ovalbumin do not respond to this antigen by the induction of IL-2 although the synthesis of other cytokines such as interferon γ and GM-CSF are up-regulated, *in vitro*. The ability of MLN cells from these tolerised mice to proliferate in the presence of the homologous antigen was also very low (Hoyne *et al*., 1993). Sun *et al.* (1999) have observed that induction of anergy in ovalbumin fed mice was accompanied by a marked decrease in MLN T cell secretion of IL-2, IL-10 and interferon γ following ovalbumin restimulation, *in vitro*. The lambs, which had been primed with the soluble larval antigen during foetal life, had a marginally higher worm count in comparison with unexposed control lambs. The *in utero* antigenic exposure of the foetal lambs seem to have modulated their immune response as

perinatal lambs to become tolerised to the parasite. The failure of the MLN from the *in utero* antigen-exposed lambs to upregulate IL-2 *in vivo* might have played a critical role in the development of this phenomenon. Thus it appears that the gut of the foetal lamb at 100 days of gestation has reached a developmental stage identical to that of the adult animals. At both ages, prior oral antigenic sensitisation followed by re-exposure to the homologous antigen results in a down-regulation of the IL-2 response in the MLN. The present study has revealed that it can occur *in vivo* as well.

There was marked expression of the IL-2 response in the MLN of control lambs, which may be part of the primary immune response to the parasite. Other investigators have observed that the IL-2 response within the MLN is up-regulated during a primary infection with gastrointestinal nematodes. Grencis *et al.* (1987) observed that, in mice, IL-2 released by the MLN was greatest during the early phase of infection with the nematode parasite *Trichinella spiralis*. Likewise, they observed that exogenous IL-2 was able to induce significant proliferation of the MLN.

Conditions following initial stimulation of T cells *in vivo* influence the nature of the effector cells generated and the nature of the lymphokines produced on restimulation (Swain *et al.*, 1991). The elevated level of IL-2 within the MLN of control lambs could have been one of the factors that skewed the immune response in this tissue to a Th1 response. The total areas staining for IgG1, IgA and IgE were considerably less (not significant statistically) in the MLN of control lambs in comparison with the *in utero* antigen-exposed lambs. Likewise, the total area staining for eosinophils was also less in the MLN of control lambs compared to the *in utero* antigen-exposed lambs. Th2 cytokines usually encourage antibody production and enhance eosinophil proliferation (Mosmann & Sad, 1996). It appears that antibody production and eosinophil proliferation were down-regulated to a considerable extent within the MLN of control lambs due to the production of IL-2.

Despite the fact that the most active parasitic infection was progressing within the jejunum, this tissue manifested the lowest expression of IL-2 in comparison with the MLN and the IPP. Other investigators have also reported a very poor induction of IL-2 by lamina propria cells after stimulation. Almeria *et*

al. (1997) observed that the lamina propria lymphocytes of cattle infected with the nematode *Ostertagia ostertagi* expressed very low levels of IL-2. In pigs the intestinal lamina propria cells fail to produce IL-2 following conA stimulation *in vitro* (Bailey *et al.*, 1998). It appears that the gut lamina propria cells from sheep, cattle and pig gut are similar in that they produce low levels of IL-2 after stimulation. In sharp contrast, the lamina propria lymphocytes from non human primates can induce large amounts of IL-2 on mitogen activation (Zeitz *et al.*, 1988). Only further investigations can reveal why the lamina propria cells from the gut of perinatal lambs express this critical cytokine at such low levels. The diminished capacity of the lamina propria cells to express IL-2 may be the result of a general decrease in T lymphocyte reactivity. Alternatively, this may indicate down-regulation of the Th1 responses which could keep in check potentially harmful cell-mediated inflammatory reactions.

TRANSFORMING GROWTH FACTOR-β

INTRODUCTION

The Transforming growth factor β (TGF-β) superfamily of growth-regulatory molecules comprise a large number of polypeptide growth factors. Members of this superfamily have been further classified on the basis of their sequence similarity into distantly and closely related groups. The distantly related group of molecules includes mullerian inhibiting substance, the activins, the inhibins and bone morphogenetic proteins, among others (Barnard *et al.*, 1993). The closely related group comprises the three different isoforms of TGF-β viz. TGF-β1, TGF-β2 and TGF-β3 (Barnard *et al.*, 1993; Omer *et al.*, 2000). TGF-β is stored intracellularly in a latent form. Biological activation of this cytokine requires the catalytic removal of the 'latency-associated protein' by plasmin or thromospondin, *in vivo*. The three isoforms of TGF-β signal through the same serine-threonine kinase type I and type II receptors and have similar, if not identical, cellular targets (Letterio & Roberts, 1998). TGF-β is also capable of binding to the matrix proteins, macroglobulins and decorins – all of which inhibit its activity and might be important in regulation of TGF-β function (Omer *et al.*, 2000).

TGF-β is produced by every leukocte lineage including lymphocytes, macrophages and dendritic cells. TGF-β is thought to control the differentiation, proliferation and activation of immune cells through both autocrine and paracrine modes (Letterio & Roberts, 1998; Omer *et al.*, 2000). TGF-β is also an important regulator of inflammation, being proinflammatory at low concentrations and anti-inflammatory at high concentrations (Omer *et al.*, 2000). In this capacity, TGF-β could be important in maintaining the balance between control and clearance of infectious organisms on the one hand and prevention of immune-mediated pathology on the other (Omer *et al.*, 2000; Sher *et al.*, 1992).

TGF-β is also thought to play critical roles in the gastrointestinal tract. In the normal murine gastrointestinal tract the expression of TGF-β is most prominent in cells located on the villous tip. Staining for this cytokine was not detectable in the crypt regions in one study, while lymphocytes in the lamina propria were occasionally positive (Barnard *et al.*, 1993).

TGF-β expression is markedly increased in pathological conditions such as inflammatory bowel disease. In patients with active ulcerative colitis and Crohn's disease the mucosa of the gut tissue expresses increased levels of TGF-β. The expression of the cytokine is localized to cells of the lamina propria with positive cells clustered towards the luminal surface of the gut (Babyatsky *et al.*, 1996).

TGF-β is thought to maintain and enhance the barrier function of the gut mucosa by countering the effects of proinflammatory cytokines . TGF-β1 shows a marked ability to reduce the capacity of interferon γ to disrupt the epithelial barrier function (Planchon *et al.*, 1999; Planchon *et al.*, 1994).

After superficial intestinal injury, the mucosal integrity is re-established by rapid migration of epithelial cells from the adjacent area by a process called restitution. TGF-β is reported to be a potent restitution promoting cytokine (Dignass & Podolsky, 1993).

There is evidence that TGF-β might be involved in immunoglobulin isotype switching within the mucosa of the gastrointestinal tract. Ginkel *et al.* (1999) analysed the B cell functions of TGF-β gene knockout mice. The most dramatic differences in the immunoglobulin isotypes was noted in the gastrointestinal tract. Faecal extracts of TGF-β knockout mice contained 9 fold less IgA and 6.9 fold more IgG than those of control mice. In the ileum and Peyer's patches of the knockout mice there was a marked decrease in IgA positive cells and an increase in IgG and IgM positive B cells. The lack of anti-inflammatory IgA and an excess of complement fixing IgG and IgM antibodies in the knockout mice is thought to promote inflammation at mucosal surfaces resulting in pulmonary and gastrointestinal tract lesions, which ultimately lead to the early death of these gene knockout mice (Ginkel *et al.*, 1999).

The gut associated lymphoid tissue is the site of tolerance induction for numerous dietary antigens. This effect is quite marked in that, mice inoculated in the submucosa of the caecum with a colon carcinoma cell line showed a more rapid tumor growth than did mice inoculated subcutaneously. The impaired anti tumor T cell response in the gut associated lymphoid tissue was attributed at least in part, to TGF-β producing $CD8^+$ T cells (Harada *et al.*, 1995).

Santos *et al.* (1993) demonstrated that antigen specific TGF-β producing cells appeared in the Peyer's patches after myelin basic protein was administered orally. Twenty four hours after one feeding of 0.25 mg myelin basic protein, there was no proliferative response to the same protein in the Peyer's patches. The myelin basic protein induced suppression was not observed in control animals that were not fed with the protein. Stimulation with myelin basic protein of the Peyer's patch T cells from myelin basic protein fed animals resulted in the secretion of TGF-β, *in vitro*. Santos *et al.* (1993) also observed that the T cells from the Peyer's patches of animals that were fed the protein orally, were able to adoptively transfer protection to experimental allergic encephalitis in other animals.

Gonnella *et al.* (1998) examined the changes in cytokine microenvironment in gut associated lymphoid tissue in response to orally administered ovalbumin in mice. By immunohistochemistry they were able to demonstrate increased levels of IL-4, IL-10 and TGF-β in the gut after feeding mice with a single low dose (0.5 mg) of ovalbumin. The increased expression of TGF-β was evident only in the interfollicular regions and the lamina propria of the Peyer's patches. The positive cells were predominantly macrophages and $CD4^+$ T cells. TGF-β could be detected in the tissues as early as 6 hours after feeding the antigen. The cytokine could still be detected at the end of 48 hours after oral antigenic exposure. Feeding mice with a single high dose of 500 mg of ovalbumin did not induce TGF-β expression within the Peyer's patches.

It is generally observed that feeding of low doses of antigen orally induces tolerance to the specific antigen. This tolerance is believed to be maintained by means of an active cellular suppression. One of the primary mechanisms of active cellular suppression is via the secretion of suppressive cytokines such as TGF-β, IL-4 and IL-10 following antigen-specific triggering. The regulatory cells responsible for suppression of cytokine secretion can migrate to lymphoid organs and suppress immune responses (Weiner, 1997; Weiner *et al.*, 1994).

TGF-β is also thought to play a vital role in maintaining the immunological balance in parasitic infections. This cytokine plays two important roles in malarial infections in mice. Early in infection it is thought to promote

Table- 12.1: Total positive area- Transforming growth factor- β1

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Ileal Peyer's patch	1517 ± 388	509 ± 143	.012

Fig-N: Large numbers of TGF-β1 lymphocytes within the IPP mucosa of *in utero* antigen-exposed lambs. Some of the positive lymphocytes are seen clustered towards the luminal end of the villi. In contrast, control animals had very few lymphocytes, or none at all, within the mucosa of the IPP.

Fig-N:

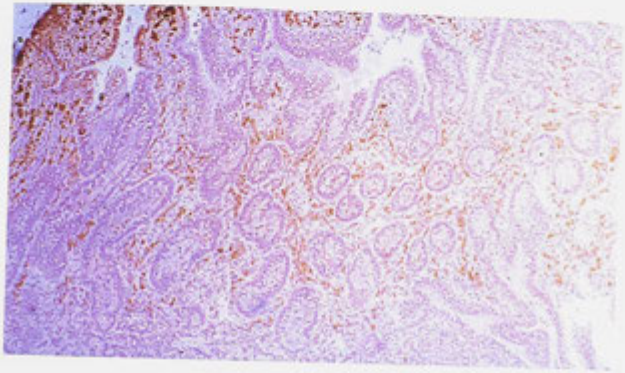
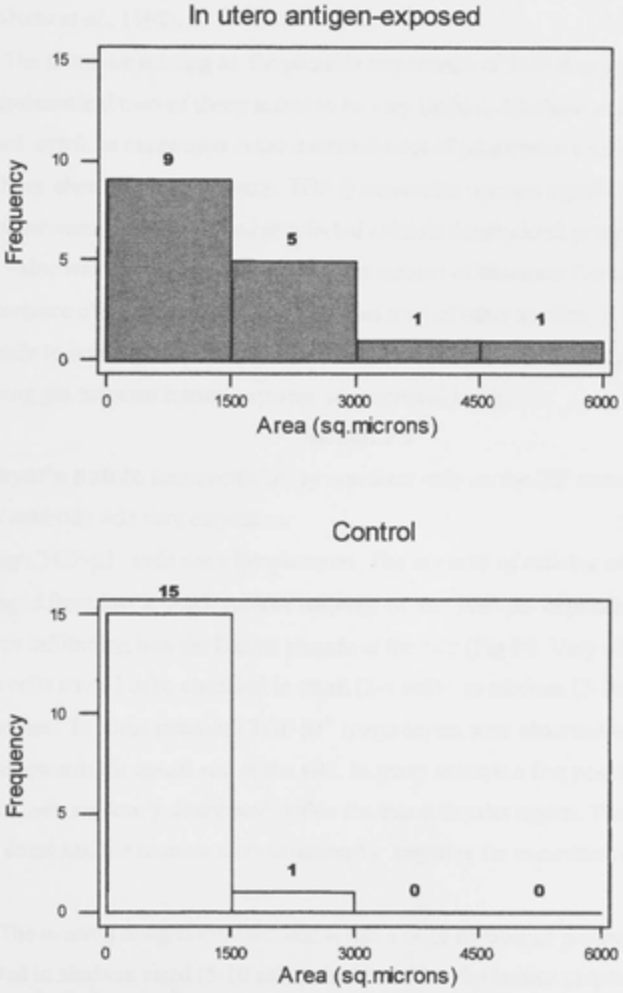


Fig-12.2: Frequency of occurrence of TGF- β1 in the IPP



Th1 mediated mechanisms that control parasite growth. In the later stages of infection, it down-regulates the production of potentially harmful pro-inflammatory cytokines (Omer *et al.*, 2000). The local injection of TGF-β enhances susceptibility to infection with normally avirulent parasite species as well as to infection with virulent parasites in genetically resistant inbred mice (Barral-Neto *et al.*, 1992).

The literature relating to the possible importance of TGF-β expression in the gastrointestinal tract of sheep seems to be very limited. Alzuherri *et al.* (1996) examined cytokine expression in the intestinal tract of paratuberculosis-infected sheep. They observed that, although TGF-β expression was not significantly different between the infected and uninfected animals the diseased group's median value was higher. With the significant amount of literature demonstrating the importance of TGF-β in the gastrointestinal tract of other species, it would be worthwhile to investigate the expression of this cytokine and its effect on the developing gut mucosal immune system of perinatal lambs.

RESULTS

Ileal Peyer's patch: Immunohistology was done only on the IPP since the primary antibody was very expensive.

Histology: TGF-β1$^+$ cells were lymphocytes. The intensity of staining of these cells ranged from low to medium. The majority of the TGF-β1 expressing cells were seen infiltrating into the lamina propria of the villi (Fig-N). Very often the positive cells tended to be clustered in small (2-4 cells) to medium (5-10 cells) sized patches. In some instances TGF-β1$^+$ lymphocytes were observed to be clustered towards the apical end of the villi. In many animals a few positive cells were also seen randomly distributed within the interfollicular region. The follicle, corona, dome and the mucosa were consistently negative for expression of TGF-β1.

The *in utero* antigen-exposed lambs had a large number of positive cells distributed in medium sized (5-10 cells) clusters within the lamina propria of the villi (Fig-N). In contrast, among the control lambs there were fewer positive lymphocytes and these tended to be either randomly distributed individual cells or small (2-4 cells) sized clusters within the lamina propria of the villi.

Image analysis: High levels of TGF-β1 were detected within the IPP of *in utero* antigen-exposed lambs. The mean TGF-β1$^+$ area was significantly greater (p=.012) in the *in utero* antigen-exposed lambs in comparison with the control group (Table-12.1).

When the area occupied by TGF-β1$^+$ cells was divided into four equal ranges and the frequency distribution of animals in each range was presented, distinct differences between the two groups became evident. In the *in utero* antigen-exposed lambs (Fig-12.2) there were seven lambs with a positive area >1500 sq.μ. In contrast, only one animal from the control group (Fig-12.2) had a positive area >1500 sq.μ. The majority of animals (15/16) from the control group had low levels (<1500 sq.μ) of TGF-β1 within the IPP, while there were only 9/16 animals in the *in utero* antigen-exposed group (Fig-12.2).

DISCUSSION

TGF-βs are a family of multifunctional cytokines. They mediate processes as diverse as development, inflammation, host defense, tissue repair, fibrosis and tumorigenesis. TGF-β1 is one of the three isoforms of TGF-β. Expression of TGF-β1 was evident in the IPP of both the groups of lambs. Most of the positive cells were observed to be infiltrating into the lamina propria of the villi. In addition, a few lymphocytes were also seen distributed within the interfollicular regions. Other regions such as the follicle, corona , dome and mucosa of the IPP were consistently negative for the expression of TGF-β1. An identical staining pattern for TGF-β has been reported in the Peyer's patches of mice after oral administration of ovalbumin (Gonnella *et al.*, 1998).

It appeared that many of the TGF-β1$^+$ cells detected might be T lymphocytes because in serial sections of the IPP in which there were very few B lymphocytes (identified by the pan B leukocyte marker CD45R), TGF-β1$^+$ lymphocytes could still be detected in large numbers. These sections contained large numbers of CD4$^+$ and CD8$^+$ T cells. Other studies have clearly established that CD4$^+$ and CD8$^+$ T lymphocytes from the Peyer's patch of mice can express TGF-β (Chen *et al.*, 1994; Santos *et al.*, 1993).

High levels of TGF-β1 were detected in the IPP of *in utero* antigen-exposed lambs. The mean area staining for TGF-β1 was significantly greater in

the *in utero* antigen-exposed group in comparison with the control lambs. The massive induction of TGF-β1 in the IPP in response to *T. colubriformis* could have been a consequence of prior oral antigenic exposure of the foetal lambs to the soluble third stage larval antigen. Oral feeding of low doses of antigen has been shown to result in the generation of antigen-specific regulatory cells. These regulatory cells, on subsequent recognition of the antigen *in vivo* or *in vitro* , secrete suppressive cytokines like TGF-β (Weiner, 1997; Weiner *et al*., 1994). In the present study, foetal lambs at 100 days of gestation were exposed to 500 micrograms of the soluble third stage larval antigen. Other investigators were able to induce tolerance against antigens fed at milligram dosage levels (Miller *et al*., 1992; Santos *et al*., 1993). This low dose antigenic exposure could have generated antigen-specific regulatory cells within the Peyer's patches of the foetal lamb. In postnatal life, after live larval challenge the secretory and excretory parasite products could have triggered pre-existing antigen specific regulatory cells in the IPP to generate TGF-β1.

Antigen-specific regulatory cells generated in the gut associated lymphoid tissue and directed against myelin basic protein have been found to have the capacity to migrate to lymphoid organs and suppress immune responses by inhibiting the generation of effector cells (Weiner, 1997; Weiner *et al*., 1994). There was significant depletion of $CD4^+$ and $CD8^+$ T lymphocytes from the IPP in the present experiment. It is possible that these T cells migrated into the jejunum, the site of parasitic infection, to carry out their effector functions. The presence of extremely high levels of TGF-β1 in the IPP could have suppressed the effector responses of these T cells. Both antigen-specific as well as bystander T cell suppression could have resulted (Weiner, 1997; Weiner *et al*., 1994). The marginally higher worm count in the *in utero* antigen-exposed lambs support this possibility.

The massive TGF-β1 expression within the IPP of *in utero* antigen-exposed lambs could also be an anti-inflammatory response generated to maintain homeostasis within the gastrointestinal tract. The marked upregulation of TNF-α a potent proinflammatory cytokine, within the jejunum and MLN of *in utero* antigen-exposed lambs was detrimental to the lambs as evident by adverse clinical symptoms and histological lesions. TGF-β has been shown to

play an essential role in down-regulating the production of potentially pathogenic proinflammatory cytokines in parasitic infections (Omer *et al.*, 2000; Sher *et al.*, 1992). An identical response could also have been generated in the present study.

NEURON SPECIFIC ENOLASE

INTRODUCTION

Neuron specific Enolase (NSE) was first isolated from the brain of cattle by Moore and McGregor (1965). It was initially reported that NSE was strictly localised in neurons and that the genes encoding for the enzyme were expressed in neuronal cells only (Schmechel *et al.*, 1978). Marangos *et al.* (1978) reported that NSE is strictly confined to neurons of the central and peripheral nervous system. NSE levels are high in areas having a high proportion of grey matter such as the cerebral cortex. It has been established that it might account for about 1.5% of the total brain soluble proteins (Marangos *et al.*, 1979).

The enolase isoenzymes catalyse the interconversion of 2 – phosphoglycerate and phosphoenol pyruvate in the glycolytic pathway. The enzyme exist as a homodimer or as a heterodimers of three subunits : alpha (Mr.46,000), beta (Mr.44,000) and gamma (Mr.46,000) . The alpha homodimeric form ($\alpha\alpha$) is the most widely distributed and is found in a variety of tissues and at extremely high concentrations (Scheuermann *et al.*, 1989). The homodimeric $\beta\beta$ and the heterodimeric $\alpha\beta$ forms are usually distributed in skeletal muscle and the heart respectively (Kato *et al.*, 1983b). The gamma homodimer ($\gamma\gamma$) is known by the common name of neuron specific Enolase (NSE). This isoform of the enzyme is found in highest concentration in neurons and tissues of neuroendocrine origin (Bishop *et al.*, 1982). It is also found in lower levels in non neural tissues as described later.

Subsequently, it became evident that a variety of different tumors of neural or neuroendocrine origin contain high levels of NSE. This feature prompted the use of NSE as a marker for such malignancies (Burke *et al.*, 1989; Pinto *et al.*, 1989). The promise of a tumor marker prompted other workers to extend their investigation to tumors of non-neural origin. Pahlman *et al.* (1986) identified NSE in non endocrine tumor specimens. Surprisingly they also observed that some of the cultured haematopoietic cells lines (T leukaemia and Epstein Barr cell lines) had NSE levels comparable to those found in some neuroblastomas and small cell carcinomas. The only differentiating feature was that neuroendocrine tumor specimens and certain tumor cell lines contained more

NSE than the non-neuroendocrine tumors. Their study proved that NSE is not exclusively confined to the nervous tissue.

Other workers soon demonstrated the ubiquitous nature of NSE. Haimoto *et al.* (1985) conclusively demonstrated the presence of NSE in normal tissue other than nervous and neuroendocrine tissue. Using immunohistochemistry they demonstrated that smooth muscle in a variety of human tissue stained strongly. Smooth muscle from the aorta, the walls of blood vessels including arteries and veins, muscularis mucosae in the gastrointestinal tract and myometrium of the uterus all stained intensely for NSE. The staining was cytoplasmic and granular in nature. NSE staining of the heart revealed that the conducting system of the heart was stained while the myocardial fibers were uniformly negative. Likewise type II alveolar epithelial cells exhibited a strong membrane staining and a weak cytoplasmic staining. Hepatocytes and pancreatic acinar cells were uniformly negative in contrast to islet cells which were strongly stained. No absorptive or crypt epithelium of the gastrointestinal tract was positive for NSE. A faintly positive reaction for the enzyme could be seen in a small number of neuroendocrine-like cells in the intraepithelial spaces of these tissues.

Spleen and lymph nodes showed similar NSE distributions. Most of the lymphocytes in the periarteriolar lymphoid sheath of the spleen, the paracortical area of the lymph nodes, and the mantle of the follicles of spleen and lymph nodes exhibited strong cytoplasmic staining (Haimoto *et al.*, 1985). Small numbers of NSE^+ cells could be detected in the centre of the lymphoid follicles of the spleen. NSE was also prominent in cells morphologically identical with plasma cells, lymphocytes and peripheral nerve fibres in the lamina propria of the duodenum (Haimoto *et al.*, 1985). Bone marrow smears contain megakaryocytes and platelets with very high levels of NSE activity. Kato *et al.* (1983a) have also demonstrated that lymphocytes contain large amounts of the three enolases. The highest level was that of the $\alpha\,\alpha$ isoform while the $\gamma\,\gamma$ form was found in the least concentration of 2.7 ng/10^6 cells.

Various methods have been used for the isolation and detection of NSE activity in tissues. Chromatography and electrophoresis were the earliest methods used. Workers like Marangos *et al.* (1978) isolated the enzyme from brain tissue by a complex protocol of column chromatography, isoelectric

focussing and gel electrophoresis. They used a direct spectrometric assay to quantify it. Radioimmunoassay and enzymatic assay for NSE have been commonly used (Marangos *et al.*, 1979). Most investigators using such techniques homogenised the tissue before the assay. This gave credibility to the criticism that the wide ranging distribution of NSE seen in non-nervous tissue was in fact an artifact arising out of the contamination of samples with nervous tissue or neuroendocrine cells (Haimoto *et al.*, 1985). This issue was addressed properly by the use of immunohistochemical techniques on fixed cryostat sections of tissues. Microscopy complemented with immunostaining was able to clearly localise the NSE activity to tissues others than neural ones (Bishop *et al.*, 1982; Haimoto *et al.*, 1985).

Most of the earlier immunohistochemical staining was carried out with well characterised, monospecific, polyclonal antisera. Their polyclonal nature did not rule out the possibility of crossreactivity or contaminating antibodies directed against the alpha or beta form. The development of monoclonal antibodies overcame this trouble (Cras *et al.*, 1986). A monoclonal antibody against NSE is available commercially. Monoclonal mouse anti-Human Neuron-Specific Enolase (DAKO™) was used for the present investigation . This antibody reacts with human $\gamma\gamma$ Enolase . The antibody does not cross react with the α or β isoforms.

The other important issue, apart from specificity, is that of the accuracy with which the enzyme can be localised in the tissue under investigation. In published reports the staining seems to be highly specific even with polyclonal sera (Bishop *et al.*, 1982; Haimoto *et al.*, 1985; Scheuermann *et al.*, 1989). It is clearly localised to specific cells in the midst of a large area of negative tissue. However, identification of the tissue or cell type becomes more difficult. When specific organs are stained identification is easier. Thus, smooth muscle in a variety of anatomical sites can be clearly identified by its NSE activity. Cells like lymphocytes can also be identified based on their morphology. However, there is no published evidence available for identifying lymphocyte sub-types by NSE activity. Based on the physical location of lymphocytes more inferences might be drawn. For example, NSE^+ cells in the paracortical region of the lymph nodes could be inferred to be T cells.

Table- 13.1: Total positive area – Neuron specific enolase

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	114 ± 21.7	262.4 ± 64.7	P=.0012
Mesenteric lymph node	269 ± 162	1484 ± 425	P=.0076
Ileal Peyer's patch	1561 ± 352	1841 ± 471	NS

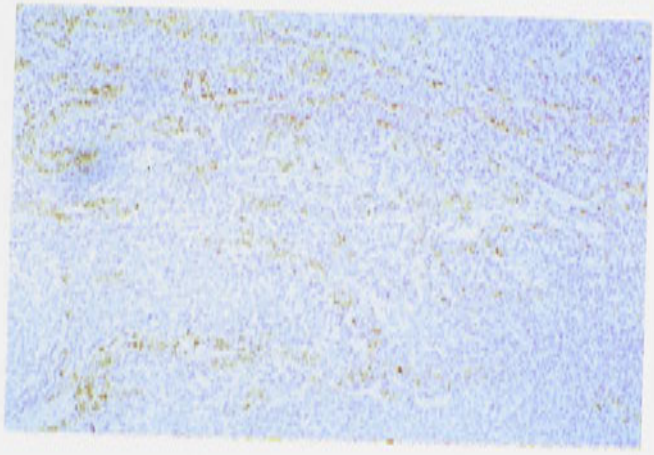
Table-13.2: Percentage of NSE^+ cells to the total leukocyte population

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	0.68 ± 0.14	1.41 ± 0.28	P=.028
Mesenteric lymph node	0.78 ± 0.5	3.69 ± 1.0	P=.0096
Ileal Peyer's patch	1.4 ± 0.36	1.7 ± 0.42	NS

Fig-O: NSE^+ lymphocytes within the MLN of *in utero* antigen-exposed lambs. The area staining for NSE was significantly lesser in the MLN of *in utero* antigen-exposed lambs in comparison with control lambs as assessed by image analysis.

Fig-P: NSE^+ lymphocytes within the MLN of control lambs. The area staining for NSE was significantly greater in the MLN of control lambs in comparison with *in utero* antigen-exposed lambs as assessed by image analysis.

Fig-O:



40 μm

Fig-P:

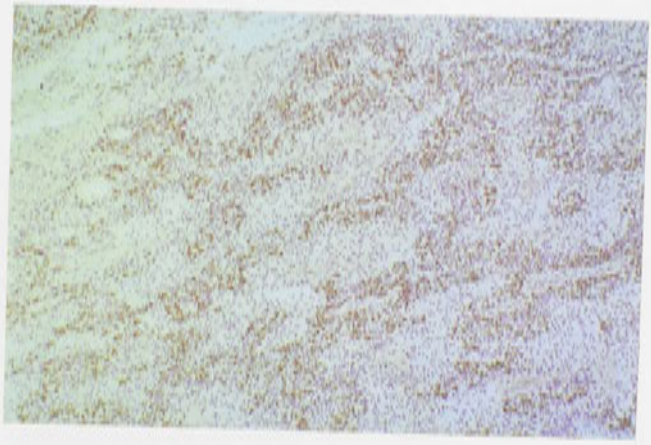
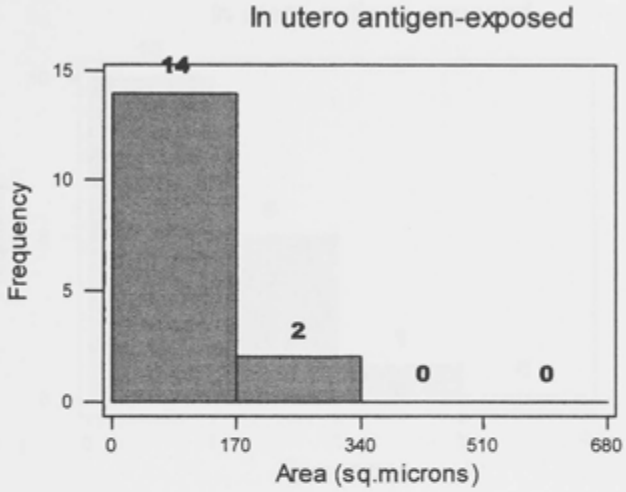


Fig-13.3: Frequency of occurrence of NSE$^+$ cells in the jejunum



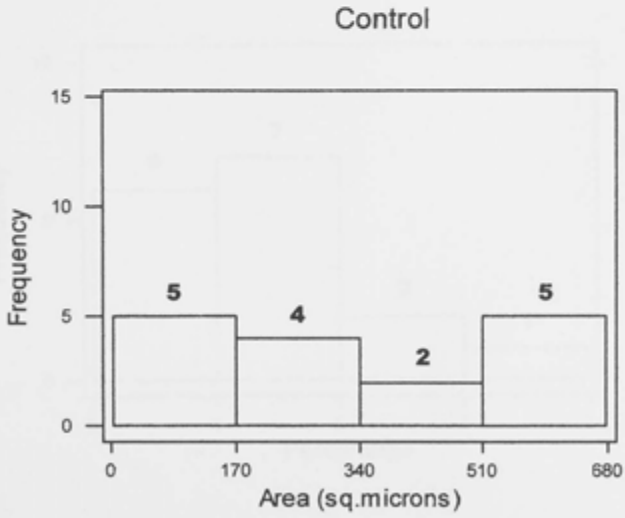
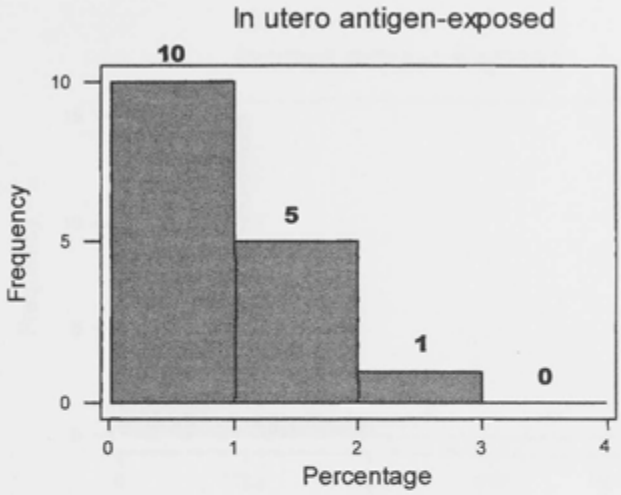


Fig- 13.4: Frequency of occurrence NSE$^+$ cells in the jejunum expressed as a percentage of total leukocyte population



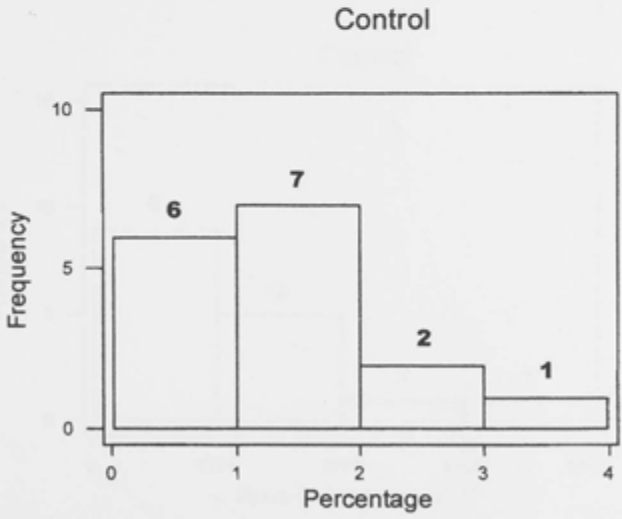


Fig-13.5: Frequency of occurrence of NSE^+ cells in the MLN

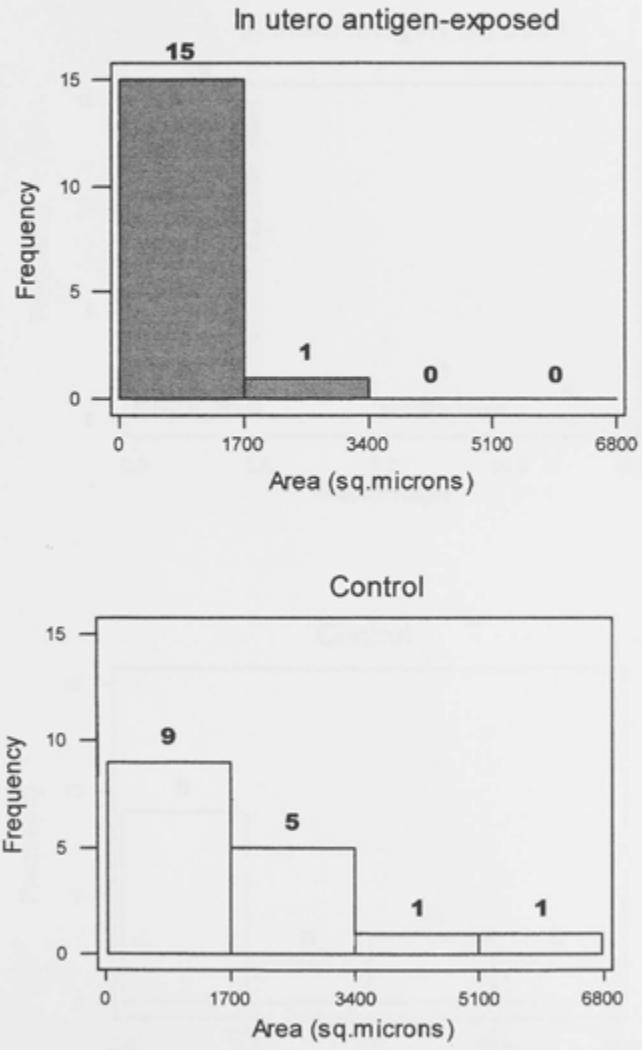
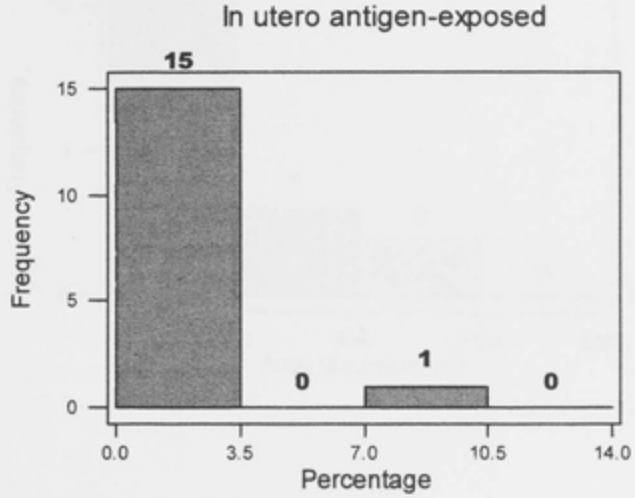


Fig- 13.6: Frequency of occurrence of NSE^+ cells in the MLN expressed as a percentage of total leukocyte population



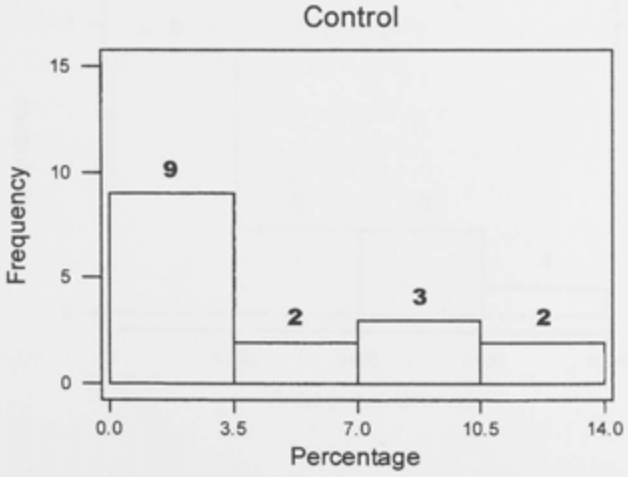
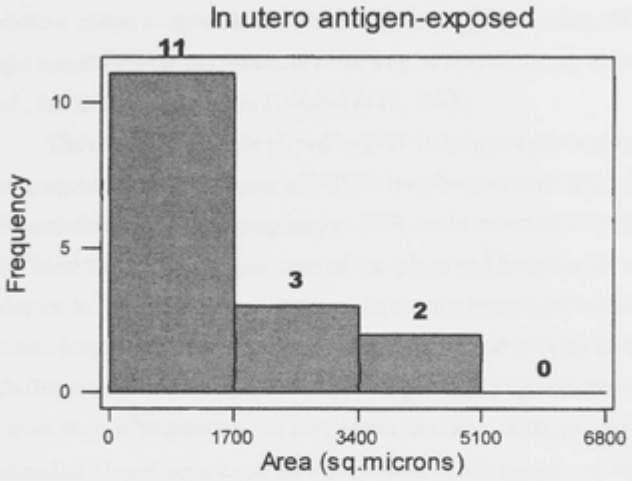
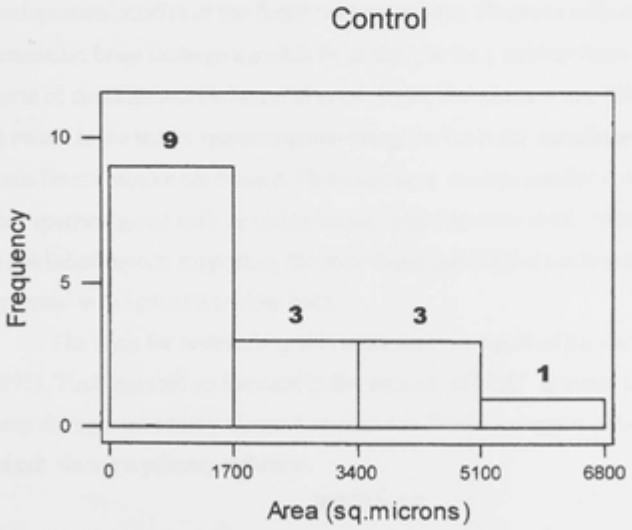


Fig-13.7: Frequency of occurrence of NSE^+ cells in the IPP





Despite all the extensive biochemical characterisation and tissue distribution studies available on this enzyme its biological role and significance remains a matter of speculation. It is thought that the expression of NSE in tissue might actually enable the tissues to sustain an additional energy demand (Bishop *et al.*, 1985; Cras *et al.*, 1986; Haimoto *et al.*, 1985).

The physiological role played by NSE in lymphocytes is not clear. It has been proposed that the presence of NSE in lymphocytes correlates with the differentiation stage of the lymphocyte. NSE can be detected in both T and B cells. Most T cells in the T cell zone of the spleen and lymph nodes are stained. However, in the B cell series, plasma cells contain significant levels of NSE whereas immature B cells which are found in lymphoid follicles contain very little (Haimoto *et al.*, 1985). Other supporting evidence for this correlation between stage of maturation and NSE expression also exists. *In vitro*, unstimulated lymphocytes contain the αα form of the isoenzyme. Following phytohaemagglutinin stimulation there is a shift to the αγ form in addition to the αα form (Rogers *et al.*, 1980). Supporting evidence also comes from developmental studies of the foetal nervous system. Neuronal cells in foetal mammalian brain undergo a switch from the α to the γ enolase form during the course of development (Schmechel *et al.*, 1980; Shinohara *et al.*, 1986). Likewise, in the testes, spermatogonia lining the basement membrane of the seminiferous tubules are stained. However, no γ enolase activity is detectable in other spermatogenic cells or seminiferous cells (Haimoto *et al.*, 1985). There are no published reports supporting the hypothesis that NSE is preferentially expressed in the gastrointestinal tract.

The basis for undertaking this study was the report of Stewart *et al.* (1995). They reported an increase in the amount of NSE^+ neurons in the gut of sheep during a secondary immune response to *Trichostrongylus colubriformis*, but not during a primary infection.

RESULTS

Histology: Staining for neuron specific enolase surprisingly did not identify the nervous innervation extending into the mucosa or lamina propria of the jejunum or ileum. Light staining of the neurons was observed only twice in 32 animals.

Interestingly, tissues other than nervous tissue were stained by this antibody. The smooth muscle and some neuronal plexus in the muscularis mucosae of the jejunum and the ileum stained strongly with this antibody. Likewise, the connective tissue capsule of the follicles in the IPP was also stained However, this staining of the smooth muscle in the muscularis as well as the connective tissue capsule was not taken into account in quantification of NSE activity in the gastrointestinal tract. Within the lamina propria and the mucosa of the gastrointestinal tract, the majority of the stained tissue had the morphological features of lymphocytes in terms of size and shape. However, some of the stained cells were larger and of varying shapes. The intensity of staining of positive cells was only moderate. However, there was sufficient contrast to differentiate the negatively stained cells from the stained ones in all regions of the gastrointestinal tract. Between the two groups of lambs there was no difference in the intensity of staining or distribution of positive cells within the tissues.

Jejunum:

Histology: The vast majority of positive cells were randomly distributed in the lamina propria and to a limited extent in the mucosal villi. A few intraepithelial cells were also stained, although very lightly, with this antibody. In the lamina propria the cells were usually found in small clusters numbering 4-8 cells or dispersed randomly. In the villi of the mucosa, the cells were usually dispersed randomly as single cells.

Image analysis: High levels of NSE positive cells could be detected in the jejunum of control animals. The mean level was significantly greater ($p=.0012$) than that in the *in utero* antigen-exposed group (Table-13.1).

When the area occupied by NSE^+ cells in the jejunum was divided into four equal quarters and correlated with the frequency distribution of the number of animals distinct differences between the two groups were apparent. In the *in utero* antigen-exposed group (Fig-13.3) all the animals had a positive area <340 sq.μ. However, in the control group (Fig-13.3) only 9/16 animals had a positive area <340 sq.μ. Likewise, in the control group of animals there were 7/16 animals with a positive area of >340 sq.μ while in the *in utero* antigen-exposed group there was none.

NSE^+ cells expressed as a percentage of the total leukocyte population: The percentage of NSE^+ cells was calculated by dividing the area occupied by NSE^+ cells by the total area stained by the pan leukocyte marker CD45. The percentage of NSE^+ cells in the jejunum (Table-13.2) was significantly greater ($p = .028$) in the control group in comparison with the *in utero* antigen-exposed lambs.

The number of animals distributed in the four quarters of NSE^+ cells expressed as a percentage of the total leukocyte population was different between the two groups. In the *in utero* antigen-exposed group 10/16 animals (Fig-13.4) had <1% of the total lymphocyte population positive for NSE. In the control group (Fig-13.4) of animals there were only six animals falling within this range (>0 and <1%). Furthermore, in the *in utero* antigen-exposed group of animals there was just one animal with NSE levels >2% in comparison to the control group where there were three animals.

Mesenteric lymph node:

Histology: In the medullary region of the MLN positive cells were found in medium (8-16 cells) clusters in the *in utero* antigen-exposed group of animals (Fig-O). However, in the control group of animal large patchy clusters (> 20 cells) of positive lymphocytes could be seen in the medullary region between the trabeculae (Fig-P). Positive cells showed a moderate intensity of staining only. There was no difference in the intensity of staining of NSE^+ lymphocytes between the two groups of lambs.

Image analysis: High levels of NSE^+ cells could be detected in the MLN of control animals. The mean level of total positive area was significantly greater ($p=.0076$) than that in the *in utero* antigen-exposed group (Table-13.1, Fig-O & P).

If the area occupied by NSE^+ cells was divided into four equal ranges and the frequency distribution of animals calculated distinct differences between the two groups became evident. In the *in utero* antigen-exposed group (Fig-13.5) the majority of animals (15/16) had a very small area (>0 and <1700 sq.μ) of NSE^+ cells. In the control group of animals (Fig-13.5) only 9/16 animals had such a small area (>0 and <1700 sq.μ) of NSE^+ cells. In the *in utero* antigen-exposed group there was only one animal with a positive area >1700sq.μ while in the control group there were seven animals.

NSE^+ cells expressed as a percentage of the total leukocyte population: The percentage of NSE^+ cells in relation to the total leukocyte population in the MLN revealed important differences between the two groups of lambs (Table-13.2). The percentage of NSE^+ cells was significantly greater in the control group ($p=.0096$) in comparison with *in utero* antigen-exposed lambs.

The frequency distribution of animals in the four quarters of NSE^+ cells expressed as a percentage of total leukocyte population was distinctly different between the two groups. In the *in utero* antigen-exposed group (Fig-13.6) the majority of animals (15/16) fell into the range with a low percentage (>0 and <3.5 %) of NSE^+ cells. In the control group (Fig-13.6) there were only nine animals that fell within this range (>0 and <3.5 %). Besides, a large number of animals (7/16) from the control group had >3.5% of NSE^+ cells while in the *in utero* antigen-exposed group there was just one animal.

Ileal Peyer's patch:

Histology: The positive cells were distributed throughout the mucosa of the IPP with a predominance in the core of the mucosal villi. Likewise, in many cases the cells tended to cluster towards the luminal tip of the villous structures. Positive intraepithelial lymphocytes could not be identified in the mucosa. The lymphocytes were found in large patchy clusters (> 20 cells) within the lamina propria of the villi. The positive lymphocytes within the mucosa were often more intensely staining than the corresponding ones found in the jejunum or MLN. Cells within the follicle were seldom stained. However, on rare occasions, the medullary region of some follicles showed positive cells which were very dark staining, irregularly shaped and large. The cells in the dome regions were not stained. Likewise, positive cells were seldom seen localised to the interfollicular region. However, very occasionally (4/32 animals) positive cells could be seen scattered throughout the interfollicular region as individual cells. There was no appreciable difference in the nature or intensity of staining between the *in utero* antigen-exposed or control groups of lambs.

Image analysis: Though not significantly different, the mean levels of NSE^+ cells in the control group tended to be marginally higher than that in the *in utero* antigen-exposed group (Table-13.1)

When the area occupied by the NSE^+ cells was divided into four equal quarters and the frequency distribution of animals calculated only minor differences between the two groups were apparent. The number of animals (Fig-13.7) having an area >3400 sq.μ was lesser (2/16) in the *in utero* antigen-exposed group while there were four animals in the control group .

NSE^+ cells expressed as a percentage of the total leukocyte population: The percentage of NSE^+ lymphocytes within the IPP showed only a marginal tendency to be higher in the control group (Table-13.2) in comparison with *in utero* antigen-exposed lambs.

DISCUSSION

The monoclonal antibody against NSE which was used did not identify neurons in the lamina propria or the villi of the mucosa of jejunum or ileum. However, innervation could be clearly demonstrated in the jejunum and ileum by immunostaining with the monoclonal antibody to CD56. Immunostaining against NSE has been used extensively for the demonstration of the innervation in the gut of a wide range of species including sheep, human, rat, pig and guinea pig (Bishop *et al.*, 1982; Karaosmanoglu *et al.*, 1996; Scheuermann *et al.*, 1989; Stewart *et al.*, 1995). However, many investigators have reported that the level of NSE immunoreactivity of enteric neurons displays wide variations. Antibodies to NSE immunostain Dogiel type II neurons particularly well, but other types of neurons are immunostained with lower intensity and some are not stained at all (Bishop *et al.*, 1985; Karaosmanoglu *et al.*, 1996; Scheuermann *et al.*, 1989). Consequently, it has not been conclusively established that NSE immunoreactivity is a marker for all enteric neurons.

The possibility that the enolase isoenzyme could have been inactivated during the processing of the tissues could be excluded in the present experiment because the tissue was directly cryosectioned without any form of tissue processing (some of which are known to inactivate sensitive and labile antigens). The fact that two animals (one from each of the control and *in utero* antigen-exposed groups) had NSE^+ innervation in the mucosa of the jejunum suggest that NSE can be expressed in the neurons of the gastrointestinal tract of young lambs. Perhaps, NSE expression was about to occur in the neurons at the time at which all of the lambs were sacrificed. However, even in these two cases the staining

intensity of neurons was quite low. It is not clear whether this lack of neuronal staining in the gut of young lambs reflects variations in the functional status of the neurons and/or an immaturity of the neurons. The results of the present study contrast with those of Stewart *et al.* (1995) who observed an increase in the number of NSE^+ neurons in the gut of adult sheep during a secondary immune response to *Trichostrongylus colubriformis*, but not during a primary infection. They have suggested that the increased numbers of NSE^+ neurons detected during the secondary immune response could be due to the increased metabolic activity of the neurons. Likewise, Stewart *et al.* (1995) believe that low levels of NSE activity observed in a primary infection could be associated with resting neurons. The age of the animals used by Stewart *et al.* (1995) was 1-2 years while in the case of the present study the lambs were 3-4 months of age. This age difference could have been one of the important factors which accounted for the difference observed in NSE activity.

NSE expression is thought to play an important role in the differentiation of neurons (Scheuermann *et al.*, 1989). It has been documented by immunocytochemistry and biochemistry of the developing rat brain that nerve cells undergo a switch from production of non neural Enolase to the NSE form of the isoenzyme, concurrently with the differentiation of post-mitotic neurons (Schmechel *et al.*, 1980). The absence of NSE staining in neurons may also reflect variations in the functional status within the nervous system. The NSE content of neurons has been found to be associated with the onset of electric activity (Cicero *et al.*, 1970). Variations in the synaptic activity or metabolic requirements of individual neurons at the time of fixation of the tissue could also account for the cell-to-cell differences in NSE staining intensity (Scheuermann *et al.*, 1989).

Clear cut immunostaining of lymphocytes was observed within the jejunum, MLN and IPP. Occasionally, larger irregularly shaped cells were also stained within the mucosa of the jejunum and IPP. Bishop *et al.* (1982) have also reported the presence of a small number of immunoreactive cells within the duodenum and jejunum. These researchers found positive cells were flask shaped and denser staining than those found within the large intestine. Since they were also positive for enteroglucagon and somatostatin they classified the

positive cells as endocrine cells. Haimoto *et al.* (1985) have also observed a small number of neuroendocrine cells staining for γ Enolase in the mucosal epithelium of the duodenum. It is possible that the larger cells identified in this study might correspond to the neuroendocrine cells that have been reported. Bishop *et al.* (1982) also observed certain NSE immunoreactive cells in the gut that could not be identified as endocrine cells due to the lack of peptides or amines. Haimoto *et al.* (1985) have clearly demonstrated the presence of positive lymphocytes within the lamina propria of the duodenum. The findings of the present study are in agreement with those of Haimoto *et al.* (1985) in that NSE^+ lymphocytes can be detected within the mucosa of the gastrointestinal tract.

The higher intensity of staining of positive cells within the IPP could possibly reflect the presence of immune cells that are in a process of induction. The positive cells within the jejunum and MLN might have already taken part in effector functions and thus might tend to stain less intensely. Bishop *et al.* (1982) have also reported a difference in the staining intensity of NSE^+ cells in different regions of the gastrointestinal tract. They observed that the cells within the small intestine stained more intensely than the ones in the large intestine. Thus variations in the intensity of staining different regions of the gastrointestinal tract seems to be the norm.

The mean levels of NSE^+ cells detected in the jejunum and MLN were significantly greater in the control group of animals. If the frequency of NSE^+ cells was expressed as a percentage of the total leukocyte population, significantly higher percentages of NSE^+ cells were apparent in the corresponding tissues in the control group in comparison with the *in utero* antigen-exposed one. Though the difference was not statistically significant, the mean levels of NSE^+ cells in the IPP also tended to be marginally higher in the control group.

In utero exposure to the parasitic antigen seems to have curtailed the expression of NSE within the cells of the gastrointestinal tract. Two important physiological attributes have been associated with the expression of NSE in cells. In cells with very high levels of metabolism, the expression of NSE may assist in sustaining the extra energy demand. To an extent this has been attributed to the chloride resistance of γ Enolase which assures the continuing activity of the

glycolytic enzyme to remain active and generate adequate ATP under adverse conditions (Cras *et al.*, 1986; Haimoto *et al.*, 1985). The expression of NSE has also been correlated with the extent of maturation of the cells. In the B cell series, plasma cells contain significant levels of NSE whereas the immature B cells found in lymphoid follicles contain very little (Haimoto *et al.*, 1985). Other supporting evidence for a correlation between stage of maturation and NSE expression exists. *In vitro,* unstimulated lymphocytes contain the α form of the isoenzyme. However, following Phytohaemagglutinin stimulation, there is a shift to the γ form of the isoenzyme (Rogers *et al.*, 1980). Active differentiation of neurons both *in vitro* (Zhang *et al.*, 1996) as well as *in vivo* (Schmechel *et al.*, 1980) correlates with an increased expression of NSE. If this evidence is taken in context the results of the present study indicate that there could have been appreciably lower levels of metabolically active tissue in the gastrointestinal tract of *in utero* antigen-exposed lambs. This evidence is also supportive of the suggestion that the number of cells that have undergone differentiation and maturation is considerably greater in the control group of animals. Thus, exposure of the foetal gut immune system to an antigen during its developmental stages might curtail the maturation of cells associated with the mucosal immune response when the animals encounters the same antigen in perinatal life.

The present study has pointed to the likely relevance of NSE^{+} cells, both lymphocytes as well as neuendocrine cells, within the gastrointestinal tract. The physiological significance of γ Enolase expression in gastrointestinal tract has not been clarified unequivocally. Even with this limited published evidence there is a strong indication that NSE might be an important immunological marker signifying the extent of maturation of the gut immune system.

WORM COUNT and FAECAL EGG COUNT

INTRODUCTION

In gastrointestinal nematodiasis the total worm count in the gut is taken as the critical measure of the degree of infestation. The presence of adult *Trichostrongylus* worms in the gut gives an indication of the number of infectious third stage larvae that were able to survive and mature into adult worms. Thus, it indirectly reflects the effectiveness of the immune response generated against the parasite by the host. In the case of lambs, the rejection of the parasite from the gut takes about nine weeks to complete (Dobson *et al.*, 1990). In the present study the final worm count that was estimated nine weeks after the infection would indicate how effective the immune responses generated by the lambs were in curtailing the development of the third stage larvae of *Trichostrongylus.*

The eggs of *Trichostrongylus colubriformis* are passed in the faeces and, under suitable conditions, hatch producing two successive non-parasitic larval stages and then the third stage or infective larvae. The passage of eggs in the faeces is thus a critical stage, essential to maintain the life cycle of the parasite. The faecal egg count is thus viewed as a parameter directly reflecting the parasitological status of the sheep. In young sheep it correlates well with the parasitic burden in the gut (Douch *et al.*, 1984). The estimation of faecal egg count has its limitations because it is influenced by a wide range of factors such as the worm burden, the sheep's immune status, variability of egg distribution within the faecal sample, the diurnal patterns of egg laying and dietary factors.

The present study was undertaken to understand the consequences of antigenic exposure of the developing gut immune during foetal life. The *in utero* antigenic exposure was not done with the intention of generating protective immunity to the parasite in postnatal life. Consequently, the two parameters namely worm count and faecal egg count were regarded as equivalent to any other parameters measured and no special significance was attached to them.

RESULTS

Worm count:

The number of lambs with adverse clinical symptoms like diarrhoea and straining were considerably higher (12/16) in the *in utero* antigen-exposed group in comparison with the control group (6/16) of lambs.

Table-14.1: Total worm count in the gut

Group	Worm count (mean ± S.E.)
In utero antigen-exposed	17334 ± 1486
Control	15581 ± 1217

Table-14.2: Faecal egg count

Time points after first larval challenge (week)	*In utero* antigen-exposed (eggs per gram)	Control (eggs per gram)
1st	5080 ± 963	5058 ± 1035
2nd	7875 ± 1533	7593 ± 1213
3rd	8419 ± 1969	7407 ± 1456
4th	6047 ± 1239	4087 ± 596
5th	5573 ± 878	4987 ± 876
6th	8042 ± 1440	6810 ± 1317

Table-14.3: Pearson correlation between total worm count and faecal egg count at different time points

Time points after first larval challenge (week)	*In utero* antigen-exposed (correlation and p value)	Control (correlation and p value)
1st	+ 0.57 (p=.09)	+ 0.32 (p=.31)
2nd	+ 0.04 (p=.89)	+ 0.33 (p=.25)
3rd	+ 0.18 (p=.50)	+ 0.57 (p=.03)*
4th	+ 0.13 (p=.64)	+ 0.56 (p=.03)*
5th	+ 0.48 (p=.07)	+ 0.62 (p=.01)*
6th	+ 0.62 (p=.03)*	+ 0.83 (p=.003)*

* Significant correlation

The total worm count in the gut was not significantly different between the two groups of lambs. However, the worm count in the *in utero* antigen-exposed group was marginally higher in comparison with the control group of lambs (Table-14.1).

Faecal egg count:

The faecal samples from the *in utero* antigen-exposed lambs had considerably more mucous covering the pellets in comparison with the faecal sample from control lambs.

The faecal egg count was not significantly different between the two groups of lambs at any of the six time points at which it was estimated. There was a tendency for the faecal egg count to be marginally higher in the *in utero* antigen-exposed group of lambs in comparison with control lambs, from the third week of larval challenge (Table-14.2).

There was very little correlation between the final worm count and the faecal egg counts at different time points in the *in utero* antigen-exposed lambs. The only time point at which the worm count had significant correlation with the faecal egg count was six weeks after the first larval challenge (Table-14.3). In contrast, there was significant correlation between the worm count and the faecal egg count at different time points after the larval challenge of the control group of lambs. The worm count was significantly correlated with the faecal egg count from the third week of larval challenge among control lambs (Table-14.3).

DISCUSSION

The faecal egg count and worm count are often considered as indicators of the effectiveness of the immune response generated against the parasite. The worm count and faecal egg counts were not significantly different between the two groups of lambs. However, the worm count and the faecal egg counts were marginally higher in the *in utero* antigen-exposed group of lambs. In addition, there was very little correlation between the worm count and the faecal egg count in the *in utero* antigen-exposed lambs in comparison with control lambs where there was significant correlation. Thus, it appears that the exposure of the developing gut immune system to the soluble larval antigen may have induced a partial tolerance to *Trichostrongylus colubriformis* in postnatal life. The different

responses observed in the gut and the peripheral blood (discussed in the preceding sections) could have possibly contributed to the marginal differences in worm and egg count observed.

OTHER MARKERS

Table- 15.1: Total positive area – CD45

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	23975.2 ± 3005.9	26167.3 ± 2422.8	NS
Mesenteric lymph node	47562.5 ± 5458.7	47722.7 ± 4652.2	NS
Ileal Peyer's patch	110953 ± 5992	109416 ± 4971	NS

Table- 15.2 : Total positive area – CD45R

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	12991.2 ± 2029.2	13614.6 ± 2739.2	NS
Mesenteric lymph node	17664.7 ± 2260.8	20730.5 ± 3229.3	NS
Ileal Peyer's patch	34650 ± 5539.8	28039.9 ± 4515.6	NS

Table- 15.3 : Total positive area – IgM

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	370.1 ± 71.4	364.2 ± 105	NS
Mesenteric lymph node	339.2 ± 41.2	419.8 ± 55.7	NS
Ileal Peyer's patch	299.8 ± 79	280.9 ± 47.5	NS

Table- 15.4 : Total positive area – IgG_1

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	1092.6 ± 233.1	1003.7 ± 242.8	NS
Mesenteric lymph node	1818 ± 380.8	1260.5 ± 223.2	NS
Ileal Peyer's patch	348.8 ± 52.8	483.6 ± 126.9	NS

Table- 15.5 : Total positive area – IgG_2

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	15.2 ± 10	39.3 ± 15.2	NS
Mesenteric lymph node	60.1 ± 17.2	36.7 ± 9.9	NS
Ileal Peyer's patch	7.15 ± 3.8	7.2 ± 3.9	NS

Table- 15.6 : Total positive area – IgA

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	1737.6 ± 259.4	1458.5 ± 264	NS
Mesenteric lymph node	374.9 ± 115	300.8 ± 38.6	NS
Ileal Peyer's patch	99.7 ± 11.3	135.8 ± 20	NS

Table- 15.7 : Total positive area – IgE

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	48.2 ± 15.2	38.7 ± 13.2	NS
Mesenteric lymph node	152.4 ± 34.2	105.5 ± 29.6	NS
Ileal Peyer's patch	60.1 ± 7.4	82.4 ± 16.9	NS

Table- 15.8 : Total positive area – MHC II

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	15233.7 ± 1994.4	16314.2 ± 1952.8	NS
Mesenteric lymph node	13755.6 ± 1506.5	17113.7± 2670.5	NS
Ileal Peyer's patch	28491.2 ± 4861.3	27486.6 ± 6457.3	NS

Table- 15.9 : Total positive area – Interleukin-1 α

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	631.1 ± 178	630.4 ± 145.5	NS
Mesenteric lymph node	1273 ± 354.9	795.7 ± 151.1	NS
Ileal Peyer's patch	1547.4 ± 284.9	1238.3 ± 234.5	NS

Table- 15.10: Total positive area – Proliferating cells nuclear antigen

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Ileal Peyer's patch	13035.8 ± 2870.6	13545.6 ± 3603.8	NS

Table- 15.11: Total positive area – CD14

Tissue	Mean ± S.E. (sq.μ)		Sig.
	In utero antigen-exposed	Control	
Jejunum	256.6 ± 59.6	352.3 ± 68.2	NS
Mesenteric lymph node	131.6 ± 20.1	169.6 ± 28.4	NS
Ileal Peyer's patch	85.5 ± 10.7	104.9 ± 17.4	NS

Table- 15.12: Mast cell numbers

Tissue	Mean ± S.E. (numbers)		Sig.
	In utero antigen-exposed	Control	
Jejunum	3.9 ± 1.3	5 ± 1.2	NS

Table- 15.13: Total antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen

Tissue	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
Jejunum	653.5 ± 326.1	159.9 ± 25.9	NS
Mesenteric lymph node	220.9 ± 65.9	157 ± 29.3	NS

Table- 15.14: Total antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen in serum

Time of collection	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
Before challenge	1866.5 ± 523.7	1029 ± 249.9	NS
4 weeks after challenge	375.3 ± 90.3	504.3 ± 156.9	NS
9 weeks after challenge	878.9 ± 363	1042.6 ± 217.5	NS

Table- 15.15: Isotype specific antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen - Jejunum

Antibody isotype	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
IgM	116.8 ± 34.5	103.1 ± 25.7	NS
IgG1	47.2 ± 21.2	32.6 ± 5.2	NS
IgG2	40.3 ± 7.9	46.8 ± 12.5	NS
IgA	67.1 ± 19.3	163.4 ± 69.7	NS
IgE	37.4 ± 9.9	40.2 ± 9.5	NS

Table- 15.16: Isotype specific antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen – Mesenteric lymph node

Antibody isotype	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
IgM	55.7 ± 8.6	83.9 ± 32	NS
IgG1	35.2 ± 16.6	25.9 ± 7.3	NS
IgG2	18.9 ± 17.6	0 ± 0	NS
IgA	31.6 ± 17.3	48 ± 24.2	NS
IgE	1.7 ± 1.3	0 ± 0	NS

Table- 15.17: Isotype specific antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen in serum before live larval challenge

Antibody isotype	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
IgM	117.3 ± 22.8	115.9 ± 33.5	NS
IgG1	945.6 ± 263.8	409.4 ± 60.7	NS
IgG2	2.3 ± 1.6	0 ± 0	NS
IgA	70.8 ± 40.3	81.6 ± 50	NS
IgE	0 ± 0	0 ± 0	NS

Table- 15.18: Isotype specific antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen in serum 4 weeks after live larval challenge

Antibody isotype	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
IgM	533.6 ± 225.3	601.3 ± 184.2	NS
IgG1	282.6 ± 112.5	194.8 ± 49.4	NS
IgG2	37.3 ± 32.5	12.6 ± 10.6	NS
IgA	208.3 ± 70.8	438.4 ± 244.2	NS
IgE	0 ± 0	0 ± 0	NS

Table- 15.19: Isotype specific antibody titre against *Trichostrongylus colubriformis* soluble third stage larval antigen in serum 9 weeks after live larval challenge

Antibody isotype	Mean ± S.E. (Titre)		Sig.
	In utero antigen-exposed	Control	
IgM	562.6 ± 99.4	541.9 ± 159.9	NS
IgG1	417.6 ± 143.1	419.1 ± 130.5	NS
IgG2	19.1 ± 15.5	0 ± 0	NS
IgA	202.9 ± 56.1	198.8 ± 88.8	NS
IgE	0 ± 0	0 ± 0	NS

GENERAL DISCUSSION

The aim of this study was to define those features of the structural and functional development of the gut-associated immune system of the perinatal lamb which are relevant to its response to parasitic infection. To achieve this, the experimental approach that was adopted entailed interfering with normal development of the gut-associated immune system and subsequently observing the impact of that interference on the responsiveness of the system. The aspect of responsiveness that was tested was the perinatal lamb's response to challenge with live larvae of a parasite to antigens from which it had been exposed in foetal life. One feature of this strategy was that experimental exposure of the foetal intestine to a parasitic antigen provided an opportunity to distinguish between the effects of normal maturation of the gut immune system dependent on gestational age and the influence of antigenic exposure on this process. In the normal course of events, the first exposure of the gut to antigens always occurs at a fixed time in development, namely full term, and so separation of these two influences is not practicable.

The selection of parameters to be measured in these experiments was intended to disclose antigenically specific and non-specific effects of exposure of the gut to parasite antigens during foetal life. The tactic employed was that of comparison of the responses, to challenge with the live parasite *Trichostrongylus colubriformis,* of two groups of lambs, one of which had been exposed to specific parasite antigens in foetal life the other being previously untreated controls. The specific objective was to measure any differences produced in the response of the immune system and the small intestine to postnatal challenge with parasite as a result of exposure to some of the parasite antigens in foetal life. The primary objective was *not* to observe the changes from normality produced by challenge.

In view of the extreme paucity of information available about the effects of exposure of the gut to antigen *in utero* and of the complexity of the responses of the gut and immune system of previously untreated lambs to parasitic infestation, no attempt was made in advance to formulate hypotheses about the nature of the processes that might be activated in either group of lambs. Specifically, it was not considered profitable to speculate in advance as to whether *in utero* exposure would produce immunity or immunological tolerance

(assuming these to be discrete entities) or whether it would confer protection or increased susceptibility in relation to challenge with live parasite. The parameters of faecal egg count and intestinal parasite burden at the time of post mortem examination were, consequently, regarded only as equivalent to any of the other measured parameters and no special significance was attached to them.

Three tissues, jejunum, ileum and mesenteric lymph nodes - were examined exhaustively after sacrifice of the two groups of lambs whilst peripheral blood and faeces were collected from each lamb on several occasions during the experiment. Examination of the jejunum was indicated because it is the location of parasite establishment and growth (Emery *et al.*, 1993; Wagland *et al.*, 1996). The ileal Peyer's patches represent the largest concentration of lymphatic tissue in the gut and also the primary site of development of B lymphocytes for the body (Griebel & Hein, 1996). The mesenteric lymph nodes are the first place of arrest of lymphocytes, antigen-presenting cells and antigenic material leaving the intestine via its draining lymphatics (Mowat & Viney, 1997).

The complexity of the gut response to parasites implies the operation of antigenically specific processes together with some others that are immunologically non-specific (Emery *et al.*, 1993). In view of this, it was anticipated that some of the responses under observation might be augmented whilst others could be diminished by exposure to parasite antigens in foetal life. Of the 22 parameters that were measured, at least 11 provided data suggesting that intervention *in utero* had modified the response of the postnatal lamb to parasitic infection. They included data on changes in the populations of different T cell phenotypes, antigen-presenting cells and eosinophils. The status of intestinal mucosal goblet cells was examined in the two groups. The levels of expression of a putative marker of lymphocyte maturation and of several cytokines believed to be relevant to the responses of lymphocytes to parasites were also monitored.

The frequency of occurrence, and distribution within the tissue, of two T lymphocyte phenotypes, $CD4^+$ and $CD8^+$ were compared in one group of lambs which had been exposed to *T. colubriformis* larval antigen *in utero* and in a second, control group that had not had any experience with that antigen before birth. Each group of lambs was orally challenged with live *T. colubriformis* at

4-6 weeks of age. There were several conspicuous differences between the two groups. Examination of the jejunum revealed that $CD4^+$ lymphocytes were preferentially clustered near the apices of the villi in the *in utero* exposed, but not in the control lambs. $CD8^+$ lymphocytes appeared to be randomly distributed throughout the lamina propria, without clustering, in both groups of lambs although image analysis disclosed that this phenotype occupied a significantly larger area in the tissues of the *in utero* antigen-exposed lambs.

Comparison of ileal tissue in the two groups of lambs revealed that both $CD4^+$ and $CD8^+$ phenotypes were responsible for occupying a significantly greater area in the control lambs. Whilst the distribution of $CD8^+$ cells was not noticeably different between the two groups of lambs, the lower content of these cells in the *in utero*, exposed lambs was apparent in the smaller interfollicular regions with loosely distributed $CD8^+$ cells. In contrast, the larger interfollicular regions in the control lambs were closely packed with $CD8^+$ cells.

A further, statistically significant difference between *in utero*-exposed and control lambs was the occurrence of a sharp decrease in the percentage of both $CD4^+$ and $CD8^+$ lymphocytes in the peripheral blood of the former group three weeks after challenge with live parasite. Without doubt, the most important feature of these results was the occurrence of significant differences in several of the parameters that were compared between the two groups. The occurrence of these differences is highly supportive of the proposition that the lymphoid tissue of foetal lamb gut is susceptible to the induction of long term modification of its immunological reactivity 50 days before full term. The other conclusion to emerge from these differences is that the response of the gut to *T. colubriformus* is quite complex. The variation in direction of the differences between the two groups when jejunum and ileum are compared bears witness to this.

The differences observed in frequency and distribution of $CD4^+$ and $CD8^+$ cells in the intestinal wall could be attributed to preferential proliferation, selective migration or, most probably, some combination of these two processes. There was no difference in the expression of 'proliferating cell nuclear antigen' by the cells of the IPP. This antigen is an essential marker for cellular DNA synthesis. This suggests that there was no preferential proliferation of cells with the ileum of either group. The observation of a significant decrease in peripheral

blood levels of both $CD4^+$ and $CD8^+$ cells following challenge of *in utero*-exposed lambs would be consistent with the withdrawal of subpopulations of these cells from the circulation. It would be reasonable to infer that this decrease reflected the retention of specifically sensitised cells in gut tissues containing parasite derived antigenic material. However, additional evidence, as for example the tracing of labelled cells, is not available.

The contrast apparent in the pattern of change in $CD8^+$ lymphocytes when jejunum and ileum are compared is notable. That the extent of tissue occupied by $CD8^+$ cells was significantly greater in lambs which had been exposed *in utero* than in controls when the jejunum was examined but significantly less in the case of ileum argues for the operation of some antigenic specificity in the observed changes. If variations in lymphocyte populations in the intestine had been initiated only as a response to inflammation, this inversion of responses in jejunum and ileum when the two groups of lambs were compared would not have been expected.

Emigration of lymphocytes from the small intestine would, in the first instance, be to the mesenteric lymph nodes whilst immigration to the intestine would be via the vasculature and, in particular, the post-capillary venules associated with organized gut lymphoid tissue. Whilst the propensity of lymphocytes originating from the gut to return selectively to that location is well established (Mackay, 1993), there is nothing to suggest that the observed imbalance of $CD8^+$ cells between jejunum and ileum reflects this process. Thus, monitoring of the mesenteric lymph nodes failed to give any indication of differences between *in utero* exposed and control lambs. The absence of any difference in mesenteric lymph node cellular composition between the two groups of lambs at the time of post mortem examination, 9 weeks after challenge with parasites, cannot exclude earlier changed that had returned to normality. The likelihood of detecting differences between the cell populations of the mesenteric lymph nodes in the two groups of lambs, as a result of differences in lymphocyte migration patterns, would depend on the length of time required for traffic through the node. If this was relatively rapid, it is likely that comparison of lymph node sections as a single time might not reveal noticeable differences despite marked variation in the number of cells migrating through the node.

The frequency and distribution of $CD5^+$ cells was also compared in the two groups of lambs. CD5 is expressed on the majority of T cells and on a small subpopulation of B cells (Chevallier *et al.*, 1998; Mackay, 1988). In contrast to all of the other markers for which results are presented, CD5 expression is an indication that cells bearing it has *not* been activated (Hopkins & Dutia, 1990). The only observed difference in $CD5^+$ cells between the two groups was that their frequency was significantly higher in the ileum of control lambs. However, rather than connoting a reduced level of activation, it is likely that this observation is attributable possibly to an increase in the size of the total T lymphocyte population in this location.

Comparison of eosinophil distribution within the intestinal tissues of *in utero* exposed and control lambs was undertaken in response to a number of previous reports associating these cells with the response to *T. colubriformis* (Bao *et al.*, 1996; Pfeffer *et al.*, 1996). Sheep infected with the parasite typically respond with an eosinophilia. Blood levels of eosinophils tend often to be universally correlated with faecal egg counts (Buddle *et al.*, 1992; Douch *et al.*, 1996b). Immunization of sheep against *Trichostrongylus* has also been observed to induce an increase in resistance to re-challenge with the parasite in association with eosinophilia (Rothwell *et al.*, 1993).

In the present experiments, clustering of eosinophils in the jejunal and ileal villi, and in the interfollicular areas of the ileal Peyer's patches, was much more evident in lambs in the control group in comparison with those in the *in utero*-exposed group. This appearance was substantiated by image analysis which revealed that the area occupied by eosinophils in the ileum of control lambs significantly exceeded that in those previously exposed to parasite antigens whilst *in utero*. Monitoring of peripheral blood eosinophil content indicated that, whilst starting levels were higher in the lambs exposed *in utero*, these did not alter significantly during the course of *T. colubriformis* infection. In contrast, a statistically significant increase in blood eosinophil levels occurred during the response of control lambs to infection. This had returned to initial levels by 8 weeks.

To the extent that an increase in eosinophil levels in blood and intestinal tissues has been reported in some studies to indicate a more effective response to

infection with *T. colubriformis* (Buddle *et al.*, 1992; Douch & Morum, 1993) and some other intestinal parasites (Gill *et al.*, 1993), the present results imply that exposure to parasite antigens whilst *in utero* served to impair the capacity of young lambs to respond to challenge with the live parasite. The results obtained failed to suggest that those animals previously exposed *in utero* had acquired resistance to the parasite.

It is of interest that, although infection with *T. colubriformis* was confined to the jejunum, increase in tissue infiltration by eosinophils occurred in the ileum of control lambs and was, quantitatively, more marked in the latter site when compared with the group of lambs exposed *in utero*. This suggests that the local response is evoked by antigen released into the gut lumen, rather than by the direct presence of the parasite in tissue, a phenomenon confined to the jejunum. Alternatively, the appearance of eosinophils throughout the small intestine may be a consequence of the systemic stimulus responsible for the eosinophilia. Presumably, this is the dissemination of parasite-derived antigens throughout the body. Immigration of eosinophils from the blood into intestinal tissues may have then occurred in response to chemotactic stimuli.

Chemotactic agents could have been introduced into the gut directly by release from the parasite and/or indirectly from T cells in response to the presence of the parasite. As regards the latter possibility, it was observed that the ileal mucosa, which was the site of the most marked increase in eosinophil infiltration in the control lambs, was also the location of the most marked concentration of $CD4^+$ and $CD8^+$ T cells. While it would be necessary to define the sequence of events by examining lambs at shorter intervals after challenge with the parasite, it is reasonable to propose that cytokine release from the lymphocytes contributed to accumulation of eosinophils.

Comparison of jejunal goblet cell frequency and morphology in control lambs and those which had been exposed to parasite antigens *in utero* revealed that there were significantly fewer cells in the latter animals and also that these cells were smaller and less frequently clustered together. Hyperplasia of goblet cells with an accompanying increase in mucin production is generally observed in association with intestinal parasite infestation (Else & Finkelman, 1998). T cells have been implicated in the mediation of these changes, possibly by means

of a Th 2 type of response (Ishikawa *et al.*, 1997). There is some evidence for an association between the strength of the goblet cell response mounted by an animal and its resistance to a parasite (Manjili *et al.*, 1998).

The curtailment of jejunal goblet cell response in lambs which had been subject to antigenic exposure *in utero* was not accompanied by any marked increase in faecal egg counts or post mortem worm counts in these animals. This implies that the contribution by goblet cells to successful elimination of the parasite is only one of a number of influences on that process and possibly represents an indirect effect on it. The significantly poorer goblet cell response by the lambs challenged *in utero* may correlate with their higher levels of TNF-α as this cytokine has been reported to depress goblet cell activity in some systems (Arnold *et al.*, 1993; Hamada *et al.*, 1991).

Examination of neuron specific enolase (NSE) in the intestinal tissues of the two groups of lambs was undertaken because of a report that cells staining for this enzyme were increased during a secondary immune response to *T. colubriformis* (Stewart *et al.*, 1995). Although originally considered to be a marker for neuronal cells, as its naming suggests, NSE has increasingly been regarded as an indication of lymphocyte differentiation, including that occurring in response to stimulation (Haimoto *et al.*, 1985; Rogers *et al.*, 1980). When applied to tissues *from T. colubriformis*-infected lambs, NSE staining was confined to cells with the morphological features of lymphocytes. Comparison of jejunal tissues from control lambs with those from animals antigenically exposed *in utero* revealed a significantly higher frequency of NSE^+ cells in the former. This was accompanied by the positive staining of a significantly greater percentage of the total leukocyte population. Similar changes were observed in the mesenteric lymph nodes, but not in the ileum.

If NSE production is taken to be an indication of differentiation by lymphocytes in response to challenge, the relative reduction in frequency of jejunal NSE^+ cells in lambs that had been exposed to parasite antigen *in utero* could be interpreted as a further indication of the depression of subsequent responsiveness by this procedure. This is especially the case given that there are indications that NSE^+ cells increase in frequency accompanying postnatal immunization against *T. colubriformis* with viable larvae. The mirroring of the

jejunal changes in the mesenteric lymph nodes is entirely consistent with the migratory pattern of cells in intestinal lymph to those nodes. The extent to which emigration of lymphocytes putatively suppressed because of the animal's experience *in utero* connotes systemic dissemination of that suppression or down-regulation is not established.

As antigen-presenting cells represent an essential component of any form of immune response to antigen, it was logical to compare their distribution in the two groups of lambs in response to challenge with *T. colubriformis*. The value of any type of observation remains dependent upon the validity of the technique applied. In the case of ovine antigen-presenting cells, two monoclonal antibodies directed against the CD1 molecule were available. SBU-T6 is believed to identify the sheep homologue of the human CD1c whereas CC20 recognizes the homologue of CD1b (Hopkins & Dutia, 1991). The former antibody recognizes most circulating B cells, monocytes and tissue macrophages in addition to cortical thymocytes and dendritic cells (Mackay *et al.*, 1985; Rhind *et al.*, 1996). CC20, in contrast, recognizes a much more restricted range of cells being confined to cortical thymocytes and dendritic cells (Dutia & Hopkins, 1991).

Quantitation of $CC20^+$ cells in the peripheral blood of lambs exposed to the parasite antigen *in utero* and control lambs revealed different patterns of response to challenge with live parasites. Whilst mean levels of $CC20^+$ were much higher in the previously exposed lambs before challenge, the levels was halved in response to parasite introduction. However, in control lambs there was a statistically significant increase in $CC20^+$ cells after challenge. These observations were consistent with a much more rapid, anamnestic response by the *in utero* exposed lambs with early retention of $CC20^+$ cells in intestinal tissues. In contrast, it seems likely that a systemic response to primary antigenic exposure was reflected in the release of $CC20^+$ cells from the tissues into the circulation.

The other significant difference in dendritic cell distribution in the two groups of lambs was an increase in cells stained with SBU-T6 in the mesenteric lymph nodes of the control group. As indicated above, this antibody detects a considerably wider range of cell types than CC20. Staining of mesenteric lymph nodes with the latter was not significantly different between the two groups of

lambs. Whilst migration of antigen-laden dendritic cells from the intestinal mucosa to the mesenteric nodes via their afferent lymphatic vessels is a well documented process, the extent to which the SBU-T6$^+$ cells in the nodes of control lambs can be equated with the CC20$^+$ cells in their ileal mucosa is uncertain. Given the broader reactivity of SBU-T6, it may be that the increased cellular content in the mesenteric nodes represented immigrant macrophages.

When the observations on CD4$^+$ and CD8$^+$ T cells are considered together with the CC20 data on dendritic cell distribution some consistent features are apparent and some paradoxical features emerge. The most consistent feature is that of significantly greater cellular responses in control lambs, a finding suggestive of a down-regulatory effect of exposure to parasite antigen in foetal life. It is paradoxical, given the location of the parasite in the jejunum that these differences in the responses of the two groups of lambs have been observed almost entirely within the ileum. The one significant jejunal difference in "conventional" immunological markers between the two groups, namely an increase in CD8$^+$ cells in these lambs which had been exposed to antigen *in utero* was not inconsistent if the CD8$^+$ phenotype of suppressor cells is taken into account.

Examination of the relative production of interleukin-1β in the two groups of lambs was suggested by previous reports incriminating it in inflammatory bowel disease (Woywodt *et al.*, 1999). This cytokine, which is generally regarded as being a component of inflammatory responses has been observed in earlier reports to be prominent both in regional lymph nodes draining inflamed tissues and in the cells trafficking in the efferent lymph from those nodes (Gohin *et al.*, 1997; Rothel *et al.*, 1997). Another stimulus in the current project to examine tissues for its presence was provided by reports that IL-1β has a role in the regulation of intestinal epithelial differentiation (Mengheri *et al.*, 1996).

Whilst most of the IL-1β$^+$ cells identified appeared to be lymphocytes, some follicular dendritic cells were also stained. These latter were notable for the intensity of their staining and also for the diffusion of the stained area into surrounding tissues, presumably reflecting release of the cytokine. IL-1β$^+$ cells were significantly more frequent in both the follicles of ileal Peyer's patches and

in the overlying mucosa of lambs in the control group. Examination of adjacent sections from the same tissues stained to identify lymphocyte phenotypes indicated that the IL-1β$^+$ cells in the follicles were likely to be overwhelmingly B cells whilst the staining mucosal cells were T cells. A similar excess of IL-1β$^+$ B cells, albeit not statistically significant, was observed when follicles in the mesenteric lymph nodes of control group lambs were compared with those of lambs that had been exposed *in utero*.

The occurrence of a decreased level of production of an inflammatory cytokine on re-exposure to the parasite *T. colubriformis* in lambs that had been previously challenged with its antigens *in utero* provided another example of the down-regulating effect of exposure by the oral route during foetal life. The reflection of the ileal changes in the draining mesenteric node suggested that the decreased response observed in the intestine would be systemically disseminated. The data relating to IL-1β expression provided the first indication of likely B cell involvement in the modification of immune responsiveness produced by *in utero* exposure to antigen. In the light of the broad range of cells (CD4$^+$ and CD8$^+$ T cells and antigen-presenting cells, not to mention eosinophils), it would seem most likely that the observed down-regulation was mediated by one or more cytokines produced by a single class of cell. If this was the case, it might be inferred that antigen specificity in recognition on re-exposure to parasite antigens resided in a single class of cell and that the observed down regulation of other types of cells was a secondary effect produced by one or more cytokines released from that single class of (presumably T) cells.

Some differences in the response of lambs exposed *in utero* and control animals were observed when the production of three other cytokines, TGF-β1 , TNF-α and IL-2, were examined. Most interestingly, the frequency of TGF-β1$^+$ cells was significantly higher, and three times the level, in the IPP of exposed lambs than it was in controls. Examination for TNF-α revealed that positive cells were present in significantly greater numbers in the jejunum of exposed lambs. MLN levels in these animals were also higher. However, TNF-α$^+$ cells were significantly more frequent in the ileal Peyer's patches of *control* lamb. The levels of IL-2$^+$ cells were relatively low in both groups of lamb, although those in

the MLN of control lambs were 50% higher than those in the corresponding tissue of lambs which had been exposed *in utero*.

Investigations of the induction of tolerance to myelin basic protein (MBP) in mice by means of oral administration has revealed the existence of an antigen-specific population of regulatory cells. These have been shown to migrate to lymphoid tissue exposed to MBP, to secrete TGF-β in response to exposure and are thought to inhibit the generation of effector T cells (Weiner, 1997). The increased presence and clustering of TGF-β^+ cells in the IPP of exposed lambs would be consistent with the indications, discussed above, that exposure to *T. colubriformis* antigen *in utero* had modified immune responsiveness and down-regulated subsequent responsiveness. TNF-α has been observed in a number of experimental situations to be produced when cells are activated. Its presence in intestinal tissues is generally associated with proinflammatory responses (Lawrence *et al.*, 1998) and, for example, it has been observed to accumulate in the MLN of rats in response to parasite infections of the gut (Ramaswamy *et al.*, 1996). The pattern of response observed in the *in utero* exposed lambs in the present experiments entailed increased presence in the jejunum (significant) and MLN, accompanied by a decrease in TNF-α^+ cells in the ileum, when compared with controls. Apart from indicating that this type of response had not been down-regulated by antigenic exposure *in utero*, this pattern raised the possibility that this earlier exposure had accentuated the capacity of that migratory cell subpopulation susceptible to activation by *T. colubriformis* to be selectively retained at, and close to the infection site. A secondary consequence of this may have been a relative depletion of the population of these cells remaining in the ileal Peyer's patches. Indications of differences in cellular migratory patterns between *in utero* exposed and control lambs following challenge with *T. colubriformis* would be consistent with the observations of relative distributions of T cells discussed above. In contrast with the suggestions of significant modification of response produced by intestinal exposure to antigen during foetal life provided by TGF-β1 and TNF-α, there appeared to be only minor IL-2 secretion responses in either group of lambs. This would be in accord with earlier reports suggesting that IL-2 secretion is not important in the response of

the intestine to nematode infections in animals like mice and cattle (Almeria *et al.*, 1997; Svetic *et al.*, 1993).

Whilst an antigen of parasite origin was selected for oral exposure of foetal lambs to be followed by challenge with live larvae in postnatal life, the present study was not intended primarily to assess the impact of the early exposure or subsequent resistance to *Trichostrongylus colubriformis*. A large volume of earlier studies has sufficed to establish the complexity of responses to intestinal parasites. As indicated in the introduction, parasite development in the two groups of lambs was regarded as being no more significant that any of the other parameters examined. Whilst faecal egg counts and final worm counts did not differ noticeably between the two groups of lambs, the clinical consequences of infection appeared to be more severe in those animals which had been exposed to parasite *in utero*.

Any future studies intended to examine specifically the impact on capacity to respond to an intestinal parasite after manipulation of the development of the foetal immune system should identify in advance the parameter which is to be taken as the primary measure of outcome. Thus, from an animal husbandry perspective, severity of clinical symptoms during the course of self-limiting infestation is likely to be more important that egg and worm counts, notwithstanding the quantitative nature of the latter.

Interpretation of the results obtained in this project has been undertaken with minimal recourse to concepts of "immunity" and "tolerance". In a situation in which a vigorous response (perhaps including a large antigenically non-specific effector component) may be detrimental for the host whilst relative unresponsiveness, with less inflammation, is less so, the terms may add little to an understanding of the process. Any overview of the modifications of responsiveness produced by exposure of foetal lambs to parasite antigen would inevitably indicate that all of the studied parameters of response to infection in perinatal life do not move in a similar direction. It may also indicate that to classify any immune response as exclusively "immune" or "tolerant" is an overly simplistic response to a complex biological phenomenon.

REFERENCES

Abrahamsen, M. S.; Lancto, C. A.; Walcheck, B.; Layton, W. & Jutila, M. A. (1997). Localization of α/β and γ/δ T lymphocytes in *Cryptosporidium parvum*-infected tissues in naive and immune calves. *Infect Immun* **65**, 2428-33.

Aleksandersen, M.; Hein, W. R.; Landsverk, T. & McClure, S. (1990). Distribution of lymphocyte subsets in the large intestinal lymphoid follicles of lambs. *Immunology* **70**, 391-397.

Allen, J. E. & Maizels, R. M. (1996). Immunology of human helminth infection. *Int Arch Allergy Immunol* **109**, 3-10.

Almeria, S.; Canals, A.; Zarlenga, D. S. & Gasbarre, L. C. (1997). Quantification of cytokine gene expression in lamina propria lymphocytes of cattle following infection with *Ostertagia ostertagi*. *J Parasitol* **83**, 1051-1055.

Alzuherri, H. M.; Woodall, C. J. & Clarke, C. J. (1996). Increased intestinal TNF-α, IL-β and IL-6 expression in ovine paratuberculosis. *Vet Immunol Immunopathol* **49**, 331-345.

Andrew, M.; Whiteley, P.; Janardhana, V.; Lobato, Z.; Gould, A. & Coupar, B. (1995). Antigen specificity of the ovine cytotoxic T lymphocyte response to blutongue virus. *Vet Immunol Immunopathol* **47**, 311-322.

Angus, K. W. & Coop, R. L. (1984). Chronic infections with *Trichostrongylus colubriformis* in lambs: influence of anthelmintic treatment on intestinal morphology and mucosal cell populations. *J Comp Pathol* **94**, 433-43.

Arai, Y.; Takanashi, H.; Kitagawa, H. & Okayasu, I. (1998). Involvement of interleukin-1 in the development of ulcerative colitis induced by dextran sulfate sodium in mice. *Cytokine* **10**, 890-896.

Arnold, J. W.; Klimpel, G. R. & Niesel, D. W. (1993). Tumor necrosis factor (TNF α) regulates intestinal mucus production during Salmonellosis. *Cell Immunol* **151**, 336-344.

Babyatsky, M. W.; Rossiter, G. & Podolsky, D. K. (1996). Expression of transforming growth factors α and β in colonic mucosa in inflammatory bowel disease. *Gastroenterology* **110**, 975-984.

Bachert, C.; Wagenmann, M. & Holtappels, G. (1998). Cytokines and adhesion molecules in allergic rhinitis. *Am J Rhinology* **12**, 3-8.

Bailey, M.; Plunkett, F.; Clarke, A.; Sturgess, D.; Haverson, K. & Stokes, C. (1998). Activation of T cells from the intestinal lamina propria of the pig. *Scand J Immunol* **48**, 177-182.

Banchereau, J. & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* **392**, 245-252.

Bao, S.; McClure, S. J.; Emery, D. L. & Husband, A. J. (1996). Interleukin-5 mRNA expressed by eosinophils and γ/δ T cells in parasite-immune sheep. *Eur J Immunol* **26**, 552-556.

Barbara, J. A. J.; Ostade, X. V. & Lopez, A. F. (1996). Tumor necrosis factor-α (TNF-α): The good, the bad and potentially very effective. *Immunol Cell Biol* **74**, 434-443.

Barnard, J. A.; Warwick, G. J. & Gold, L. I. (1993). Localization of transforming growth factor β isoforms in the normal murine small intestine and colon. *Gastroenterology* **105**, 67-73.

Barral-Neto, M.; Barral, A.; Twardzick, D.; Ellingsworth, L. & Reed, S. (1992). Transforming growth factor-β production: an important *Leishmania* virulence mechanism. *Faseb J* **6**, A1687.

Battistini, L.; Fischer, F. R.; Raine, C. S. & Brosnan, C. F. (1996). CD1b is expressed in multiple sclerosis lesions. *J Neuroimmunol* **67**, 145-151.

Bauch, A.; Campbell, K. S. & Reth, M. (1998). Interaction of the CD5 cytoplasmic domain with the Ca^{2+}/calmodulin- dependent kinase IIδ. *Eur J Immunol* **28**, 2167-2177.

Begara-McGorum, I.; Wildblood, L. A.; Clarke, C. J.; Connor, K. M.; Stevenson, K.; McInnes, C. J.; Sharp, J. M. & Jones, D. G. (1998). Early immunopathological events in experimental ovine paratuberculosis. *Vet Immunol Immunopathol* **63**, 265-287.

Beh, K. J. (1987). Production and characterization of monoclonal antibodies specific for sheep IgG_1 and IgG_2. *Vet Immunol Immunopathol* **14**, 187-196.

Belley, A.; Keller, K.; Goettke, M. & Chadee, K. (1999). Intestinal mucins in colonization and host defense against pathogens. *Am J Trop Med Hyg* **60**, 10-15.

Ben-Sasson, S. A.; Gros, G. L.; Conrad, D. H.; Finkelman, F. D. & Paul, W. E. (1990). IL-4 production by T cells from naive donors. IL-2 is required for IL-4 production. *J Immunol* **145**, 1127-1131.

Birkebak, T. A.; Palmer, G. H.; Davis, W. C. & McElwain, T. F. (1994). Quantitative characterization of the CD5 bearing lymphocyte population in the peripheral blood of normal sheep. *Vet Immunol Immunopathol* **41**, 181-186.

Bishop, A. E.; Carlei, F.; Lee, V.; Trojanowski, J.; Marangos, P. J.; Dahl, D. & Polak, J. M. (1985). Combined immunostaining of neurofilaments, neuron specific enolase, GFAP and S-100 : A possible means for assessing the morphological and functional status of the enteric nervous system. *Histochemistry* **82**, 93-97.

Bishop, A. E.; Polak, J. M.; Facer, P.; Ferri, G.-L.; Marangos, P. J. & Pearse, A. G. E. (1982). Neuron specific enolase: A common marker for the endocrine cells and innervation of the gut and pancreas. *Gastroenterology* **83**, 902-915.

Bloom, P. D. & Boedeker, E. C. (1996). Mucosal immune responses to intestinal bacterial pathogens. *Semi Gastrointestinal Dis* **7**, 151-166.

Bode, H.; Schmitz, H.; Fromm, M.; Scholz, P.; Riecken, E. O. & Schulzke, J. D. (1998). IL-1β and TNF-α, but not IFN-α, IFN-γ, IL-6 or IL-8, are secretory mediators in human distal colon. *Cytokine* **10**, 457-65.

Braciale, T. J. (1979). Immunological recognition of influenza virus-infected cells I. Generation of a virus strain-specific and a cross-reactive subpopulation of cytotoxic T cells in response to type A influenza viruses of different subtypes. *Cell Immunol* **33**, 423-436.

Brockhaus, M.; Schoenfeld, H.-J.; Schlaeger, E.-J.; Hunziker, W.; Lesslauer, W. & Loetscher, H. (1990). Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc Natl Acad Sci USA* **87**, 3127-3131.

Brown, W. C.; Rice-Ficht, A. C. & Estes, D. M. (1998). Bovine type 1 and type 2 responses. *Vet Immunol Immunopathol* **63**, 45-55.

Buddle, B. M.; Jowett, G.; Green, R. S.; Douch, P. G. & Risdon, P. L. (1992). Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *Int J Parasitol* **22**, 955-960.

Burke, A. P.; Sobin, L. H.; Federspiel, B. H. & Shekitka, K. M. (1989). Appendiceal carcinoids: correlation of histology and immunohistochemistry. *Mod Pathol* **2**, 630-637.

Burrells, C.; Clarke, C. J.; Colston, A.; Kay, J. M.; Porter, J.; Little, D. & Sharp, J. M. (1999). Interferon-γ and interleukin-2 release by lymphocytes derived from the blood, mesenteric lymph nodes and intestines of normal sheep and those affected with paratuberculosis (Johne's disease). *Vet Immunol Immunopathol* **68**, 139-48.

Burrells, C.; Nettleton, P. F.; Reid, H. W.; Miller, H. R. P.; Hopkins, J.; McConnell, I.; Gorrell, M. D. & Brandon, M. R. (1989). Lymphocyte

subpopulations in the blood of sheep persistently-infected with Border Disease virus. *Clin Exp Immunol* **76**, 446-451.

Butch, A. W.; Chung, G.-H.; Hoffmann, J. W. & Nahm, M. H. (1993). Cytokine expression by germinal centre cells. *J Immunol* **150**, 39-47.

Canals, A.; Zarlenga, D. S.; Almeria, S. & Gasbarre, L. C. (1997). Cytokine profile induced by a primary infection with *Ostertagia ostertagi* in cattle. *Vet Immunol Immunopathol* **58**, 63-75.

Cerretti, D. P.; Kozlosky, C. J.; Mosley, B.; Nelson, N.; Van Ness, K.; Greenstreet, T. A.; March, C.; Kornheim, S. R.; Druck, T.; Cannizzaro, L. A.; Huebner, K. & Black, R. A. (1992). Molecular cloning of interleukin-1 β converting enzyme. *Science* **256**, 97-100.

Chen, Y.; Inoble, J.-I.; Marks, R.; Gonnella, P. & Kuchroo, V. K. (1995). Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* **376**, 177-180.

Chen, Y.; Kuchroo, V. K.; Inoble, J.-I.; Hafler, D. A. & Weiner, H. L. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* **265**, 1237-1240.

Cheng, H. & Leblond, C. P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. IV. Unitarian theory of the origin of the four epithelial cell types. *Am J Anat* **141**, 537-561.

Chevallier, N.; Berthelemy, M.; Laine, V.; Le Rhun, D.; Femenia, F.; Polack, B.; Naessens, J.; Levy, D. & Schwartz-Cornil, I. (1998). B-1-like cells exist in sheep. Characterization of their phenotype and behaviour. *Immunology* **95**, 178-184.

Cicero, T.; Cowan, W. M. & Moore, B. W. (1970). Changes in the concentration of two brain proteins S-100 and 14-3-2' during the development of the avian optic tectum. *Brain Res* **24**, 1-10.

Conner, G. H.; Richardson, M. & Carter, G. R. (1973). Prenatal immunisation and protection of newborn: ovine and bovine foetuses vaccinated with *Escherichia coli* antigens by the oral route and exposed to challenge inoculum at birth. *Amer J vet Res* **34**, 737-741.

Cras, P.; Federsppiel, S. S.; Gheuens, J.; Martin, J.-J. & Lowenthal, A. (1986). Demonstration of neuron-specific enolase in nonneural tumors using a specific monoclonal antibody. *Ann Neurology* **20**, 106-107.

Cuzic, S.; Ritz, E. & Waldherr, R. (1992). Dendritic cells in glomerulonephritis. *Virchows Arch B Cell Pathol Incl Mol Pathol* **62**, 357-63.

Debard, N.; Sierro, F. & Kraehenbuhl, J. P. (1999). Development of Peyer's patches, follicle-associated epithelium and M cell: lessons from immunodeficient and knockout mice. *Semin Immunol* **11**, 183-191.

Dignass, A. U. & Podolsky, D. K. (1993). Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor β. *Gastroenterology* **105**, 1323-1332.

Dinarello, C. A. (1991). Interleukin-1 and interleukin-1 antagonism. *Blood* **77**, 1627-1652.

Dinarello, C. A. (1992). Role of Interleukin-1 in infectious diseases. *Immunol Rev* **127**, 119-146.

Dobson, R. J.; Waller, P. J. & Donald, A. D. (1990). Population dynamics of *Trichostrongylus colubriformis* in sheep: the effect of infection rate on the establishment of infective larvae and parasite fecundity. *Int J Parasitol* **20**, 347-352.

Douch, P. G.; Green, R. S.; Huntley, J. F. & Risdon, P. L. (1996a). Serum mast cell proteinase responses of sheep to challenge with *Trichostrongylus colubriformis* and the effect of dexamethasone treatment. *Int J Parasitol* **26**, 91-95.

Douch, P. G.; Green, R. S.; Morris, C. A.; McEewan, J. C. & Windon, R. G. (1996b). Phenotypic markers for selection of nematode-resistant sheep. *Int J Parasitol* **26**, 899-911.

Douch, P. G.; Green, R. S. & Risdon, P. L. (1994). Antibody responses of sheep to challenge with *Trichostrongylus colubriformis* and the effect of dexamethasone treatment. *Int J Parasitol* **24**, 921-928.

Douch, P. G.; Harrison, G. B.; Elliott, D. C.; Buchanan, L. L. & Greer, K. S. (1986). Relationship of gastrointestinal histology and mucus antiparasite activity with the development of resistance to *Trichostrongyle* infections in sheep. *Vet Parasitol* **20**, 315-331.

Douch, P. G. & Morum, P. E. (1993). The effect of age on the response of Romney sheep to gastrointestinal nematodes during grazing. *Int J Parasitol* **23**, 651-655.

Douch, P. G. C.; Harrison, G. B. L.; Buchanan, L. L. & Brunsdon, R. V. (1984). Relationship of histamine in tissues and antiparasitic substances in gastrointestinal mucus to the development of resistance to *Trichostrongylus* infection in young sheep. *Vet Parasitol* **16**, 273-288.

Douch, P. G. C.; Harrison, G. B. L.; Buchanan, L. L. & Greer, K. S. (1983). *In vitro* bioassay of sheep gastrointestinal mucus for nematode paralysing activity mediated by substance with some properties characteristic of SRS-A. *Int J Parasitol* **13**, 207-212.

Doyle, C. & Strominger, J. L. (1987). Interaction between CD4 and class II molecules mediates cell adhesion. *Nature* **330**, 256-259.

Duchmann, R.; Schmitt, E.; Knolle, P.; Meyer zum Buschenfelde, K. H. & Neurath, M. (1996). Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by interleukin-10 or antibodies to interleukin-12. *Eur J Immunol* **26**, 934-938.

Dutia, B. M. & Hopkins, J. (1991). Analysis of the CD1 cluster in sheep. *Vet Immunol Immunopathol* **27**, 189-194.

Egan, P. J.; Andrews, A. E.; Barcham, G. J.; Brandon, M. R. & Nash, A. D. (1994a). Production and application of monoclonal antibodies to ovine interleukin-1 α and interleukin-1 β. *Vet Immunol Immunopathol* **41**, 241-257.

Egan, P. J.; Rothel, J. S.; Andrews, A. E.; Seow, H.-F.; Wood, P. R. & Nash, A. D. (1994b). Characterization of monoclonal antibodies to ovine tumor necrosis factor-α and development of a sensitive immunoassay. *Vet Immunol Immunopathol* **41**, 259-274.

Eigler, A.; Sinha, B.; Hartmann, G. & Endres, S. (1997). Taming TNF: strategies to restrain this proinflammatory cytokine. *Immunol Today* **18**, 487-492.

Else, K. J. & Finkelman, F. D. (1998). Intestinal nematode parasites, cytokines and effector mechanisms. *Int J Parasitol* **28**, 1145-1158.

Emery, D. L.; McClure, S. J.; Davey, R. J. & Bendixsen, T. (1999). Induction of protective immunity to *Trichostrongylus colubriformis* in neonatal merino lambs. *Int J Parasitol* **29**, 1037-1046.

Emery, D. L.; McClure, S. J. & Wagland, B. M. (1993). Production of vaccines against gastrointestinal nematodes of livestock. *Immunol Cell Biol* **71**, 463-472.

Emile, J. F.; Fraitag, S.; Leborgne, M.; de Prost, Y. & Brousse, N. (1994). Langerhans' cell histiocytosis cells are activated Langerhans' cells. *J Pathol* **174**, 71-76.

Finkelman, F. D.; Pearce, E. J.; Urban, J. F. J. & Sher, A. (1991). Regulation and biological function of helminth-induced cytokine responses. *Parasitol Today*, A62-A66.

Finkelman, F. D.; Shea-Donohue, T.; Goldhill, J.; Sullivan, C. A.; Morris, S. C.; Madden, K. B.; Gause, W. C. & Urban, J. F. (1997). Cytokine regulation of host defense against parasitic gastrointestinal nematodes: Lessons from studies with rodent models. *Annu Rev Immunol* **15**, 505-533.

Fitch, F. W.; McKisic, M. D.; Lancki, D. W. & Gajewski, T. F. (1993). Differential regulation of murine lymphocyte subset. *Annu Rev Immunol* **11**, 29-48.

Freedman, A. S.; Freeman, G.; Whitman, J.; Segil, J.; Daley, J. & Nadler, M. L. (1988). Pre-exposure of human B cells to recombinant IL-1 enhances subsequent proliferation. *J Immunol* **141**, 3398-3404.

Fujieda, S.; Sieling, P. A.; Modlin, R. L. & Saxon, A. (1998). CD1-restricted T-cells influence IgG subclass and IgE production. *J Allergy Clin Immunol* **101**, 545-551.

Garlisi, C. G.; Falcone, A.; Kung, T. T.; Stelts, D.; Pennline, K. J.; Beavis, A. J.; Smith, S. R.; Egan, R. W. & Umland, S. P. (1995). T cells are necessary for Th2 cytokine production and eosinophil accumulation in airways of antigen-challenged allergic mice. *Clin Immunol Immunopathol* **75**, 75-83.

Garside, P.; Grencis, R. K. & Mowat, A. M. (1992). T lymphocyte dependent enteropathy in murine *Trichinella spiralis* infection. *Parasite Immunol* **14**, 217-225.

Gerber, H. A.; Morris, B. & Trevella, W. (1986). The role of gut-associated lymphoid tissues in the generation of immunoglobulin-bearing lymphocytes in sheep. *Aust J Exp Biol Med Sci* **64**, 201-213.

Gerdes, J.; Stahmer, I.; Flad, H.-D. & Pileri, S. (1990). Follicular dendritic cells and human immunodeficiency infection. *Local Immunity* **6**, 159-165.

Gibson, T. E. & Parfitt, J. W. (1972). The effect of age on the development by sheep of resistance to *Trichostrongylus colubriformis*. *Res Vet Sci* **13**, 529-535.

Gill, H. S.; Watson, D. L. & Brandon, M. R. (1993). Monoclonal antibody to $CD4^+$ T cells abrogates genetic resistance to *Haemonchus contortus* in sheep. *Immunology* **78**, 43-49.

Ginkel, F. W. V.; Wahl, S. M.; Kearney, J. F.; Kweon, M.; Fujihashi, K.; Burrows, P. D.; Kiyono, H. & McGhee, J. R. (1999). Partial IgA-deficency with increased Th2-type cytokines in TGF-β knockout mice. *J Immunol* **163**, 1951-1957.

Gohin, I.; Olivier, M.; Lantier, I.; Pepin, M. & Lantier, F. (1997). Analysis of the immune response in sheep efferent lymph during *Salmonella abortus ovis* infection. *Vet Immunol Immunopathol* **60**, 111-130.

Gonnella, P. A.; Chen, Y.; Inobe, J.; Komagata, Y.; Quartulli, M. & Weiner, H. L. (1998). In situ immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice. *J Immunol* **160**, 4708-4718.

Gorrell, M. D.; Miller, H. R. & Brandon, M. R. (1988a). Lymphocyte phenotypes in the abomasal mucosa of sheep infected with *Haemonchus contortus*. *Parasite Immunol* **10**, 661-674.

Gorrell, M. D.; Willis, G.; Brandon, M. R. & Lascelles, A. K. (1988b). Lymphocyte phenotypes in the intestinal mucosa of sheep infected with *Trichostrongylus colubriformis*. *Clin Exp Immunol* **72**, 274-279.

Gray, P. W.; Glaister, D.; Chen, E.; Goeddel, D. V. & Pennica, D. (1986). Two interleukin-1 genes in the mouse: cloning and expression of the cDNA for murine interleukin-1 β. *J Immunol* **137**, 3644-3648.

Grell, M.; Douni, E.; Wajant, H.; Lohden, M.; Clauss, M.; Maxeiner, B.; Georgopoulous, S.; Lesslauer, W.; Kollias, G.; Pfizenmaier, K. & Scheurich, P. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80KDa tumor necrosis factor receptor. *Cell* **83**, 793-802.

Grencis, R. K.; Riedlinger, J. & Wakelin, D. (1987). Lymphokine production by T cells generated during infection with *Trichinella spiralis*. *Int Arch Allergy Appl Immunol* **83**, 92-95.

Griebel, P. & Ferrari, G. (1995). CD40 signaling in ileal Peyer's patch B cells: implications for T cell-dependent antigen selection. *Int Immunol* **7**, 369-379.

Griebel, P. J. & Hein, W. R. (1996). Expanding the role of Peyer's patches in B-cell ontogeny. *Immunol Today* **17**, 30-39.

Gunnes, G.; Press, C. M.; Tverdal, A. & Landsverk, T. (1998). Compartments within the lymph node cortex of calves and adult cattle differ in the distribution of leukocyte populations: an immunohistochemical study using computer-assisted morphometric analysis. *Dev Comp Immunol* **22**, 111-123.

Gupta, V. K.; McConnell, I.; Dalziel, R. G. & Hopkins, J. (1996). Identification of the sheep homologue of the monocyte cell surface molecule-CD14. *Vet Immunol Immunopathol* **51**, 89-99.

Gyorffy, E. J.; Glogauer, M.; Kennedy, L. & Reynolds, J. D. (1992). T-cell receptor-γδ association with lymphocyte populations in sheep intestinal mucosa. *Immunology* **77**, 25-30.

Haig, D. M.; Stevenson, L. M.; Thomson, J.; Percival, A. & Smith, W. D. (1995). Haemopoietic cell responses in the blood and bone marrow of sheep infected with the abomasal nematode *Telodorsagia circumcincta*. *J Comp Pathol* **112**, 151-164.

Hailata, N.; Johnson, R.; Al-Bagdadi, F. & Hanash, S. (1995). Proliferating cell nuclear antigen expression in sheep infected with bovine leukemia virus. *Vet Immunol Immunopathol* **44**, 211-222.

Haimoto, H.; Takahashi, Y.; Koshikawa, T.; Nagura, H. & Kato, K. (1985). Immunohistochemical localization of γ-enolase in normal human tissues other than nervous and neuroendocrine tissues. *Lab Invest* **52**, 257-263.

Hall, J. G.; Hopkins, J. & Orlans, E. (1977). Studies on the lymphocytes of sheep III: Destination of lymph-borne immunoblasts in relation to their tissue of origin. *Eur J Immunol* **7**, 30-37.

Hamada, Y.; Kobayashi, K. & Brown, W. R. (1991). Tumor necrosis factor-α decreases expression of the intestinal IgG Fc binding site by HT29-N2 cells. *Immunology* **74**, 298-303.

Harada, M.; Matsunaga, K.; Oguchi, Y.; Iijima, H.; Ito, O.; Tamada, K.; Kimura, G. & Nomoto, K. (1995). The involvement of transforming growth factor β in the impaired antitumor T-cell response at the gut-associated lymphoid tissue (GALT). *Cancer Res* **55**, 6146-6151.

Hein, W. R. (1999). DU12-24-3 : Sheep eosinophil marker. Personal communication.

Hein, W. R.; Dudler, L. & Mackay, C. R. (1989). Surface expression of differentiation antigens on lymphocytes in the ileal and jejunal Peyer's patches of lambs. *Immunology* **68**, 365-370.

Hir, M. L.; Bluethmann, H.; Kosco-Vilbois, M. H.; Muller, M.; Padova, F. D.; Moore, M.; Ryffel, B. & Eugster, H.-P. (1996). Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling. *J Exp Med* **183**, 2367-2372.

Hohenhaus, M. A.; Josey, M. J.; Dobson, C. & Outteridge, P. M. (1998). The eosinophil leukocyte, a phenotypic marker of resistance to nematode parasites, is associated with calm behaviour in sheep. *Immunol Cell Biol* **76**, 153-158.

Hohmann, H. P.; R., R.; Brockhaus, M. & Van Loon, A. P. G. M. (1989). Two different cell types have different major receptors for human tumor necrosis factor (TNF-α). *J Biol Chem* **264**, 14927-14934.

Hopkins, J. (1991). Workshop studies on the ovine CD4 homologue. *Vet Immunol Immunopathol* **27**, 101-102.

Hopkins, J. & Dutia, B. M. (1990). Monoclonal antibodies to the sheep analogues of human CD45 (leukocyte common antigen), MHC class I and CD5. Differential expression after lymphocyte activation *in vivo*. *Vet Immunol Immunopathol* **24**, 331-346.

Hopkins, J. & Dutia, B. M. (1991). Workshop studies on the ovine CD1 homologue. *Vet Immunol Immunopathol* **27**, 97-99.

Howard, C. J.; Sopp, P.; Bembridge, G.; Young, J. & Parsons, K. R. (1993). Comparison of CD1 monoclonal antibodies on bovine cells and tissues. *Vet Immunol Immunopathol* **39**, 77-83.

Hoyne, G. F.; Callow, M. G.; Kuhlman, J. & Thomas, W. R. (1993). T-cell lymphokine response to orally administered proteins during priming and unresponsiveness. *Immunology* **78**, 534-540.

Huntley, J. F.; Patterson, M.; Mackellar, A.; Jackson, F.; Stevenson, L. M. & Coop, R. L. (1995). A comparison of the mast cell and eosinophil responses of sheep and goats to gastrointestinal nematode infections. *Res Vet Sci* **58**, 5-10.

Husband, A. J. & McDowell, G. H. (1975). Local and systemic immune responses following oral immunisation of foetal lambs. *Immunology* **29**, 1019-1028.

Ishikawa, N.; Horii, Y. & Nawa, Y. (1993). Immune-mediated alteration of the terminal sugars of goblet cell mucins in the small intestine of *Nippostrongylus brasiliensis*-infected rats. *Immunology* **78**, 303-307.

Ishikawa, N.; Horii, Y.; Oinuma, T.; Suganuma, T. & Nawa, Y. (1994). Goblet cell mucins as the selective barrier for the intestinal helminths: T-cell-independent alteration of goblet cell mucins by immunologically 'damaged' *Nippostrongylus brasiliensis* worms and its significance on the challenge infection with homologous and heterologous parasites. *Immunology* **81**, 480-486.

Ishikawa, N.; Wakelin, D. & Mahida, Y. R. (1997). Role of T helper 2 cells in intestinal goblet cell hyperplasia in mice infected with *Trichinella spiralis*. *Gastroenterology* **113**, 542-549.

Iwasaki, A. & Kelsall, B. L. (1999). Mucosal immunity and inflammation. I. Mucosal dendritic cells: their specialized role in initiating T cell responses. *Am J Physiol* **276**, G1074-G1078.

Jones, W. O.; Emery, D. L.; McClure, S. J. & Wagland, B. M. (1994). Changes in inflammatory mediators and larval inhibitory activity in intestinal contents and mucus during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *Int J Parasitol* **24**, 519-525.

Josefsen, R. D. & Landsverk, T. (1996). T cell subsets and langerhans cells in the forestomach mucosa of adult sheep and sheep foetuses. *Vet Immunol Immunopathol* **51**, 101-111.

Kambara, T. & McFarlane, R. G. (1996). Changes in T cell subpopulations of sheep due to age and dietary protein intake: association with protective immunity in *Trichostrongylus colubriformis*. *Vet Immunol Immunopathol* **51**, 127-135.

Kambara, T.; McFarlane, R. G.; Abell, T. J.; McAnulty, R. W. & Sykes, A. R. (1993). The effect of age and dietary protein on immunity and resistance in lambs vaccinated with *Trichostrongylus colubriformis*. *Int J Parasitol* **23**, 471-476.

Kaouass, M.; Deloyer, P.; Gouders, I.; Peulen, O. & Dandrifosse, G. (1997). Role of interleukin-1 β, interleukin-6, and TNF-α in intestinal maturation induced by dietary spermine in rats. *Endocrine* **6**, 187-194.

Karanu, F. N.; McGuire, T. C.; Davis, W. C.; Besser, T. E. & Jasmer, D. P. (1997). $CD4^+$ T lymphocytes contribute to protective immunity induced in sheep and goats by *Haemonchus contortus* gut antigens. *Parasite Immunol* **19**, 435-445.

Karaosmanoglu, T.; Aygun, B.; Wade, P. R. & Gershon, M. D. (1996). Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. *Anat Rec* **244**, 470-480.

Kato, K.; Asai, R.; Shimizu, A.; Suzuki, F. & Ariyoshi, Y. (1983a). Immunoassays of three enolase isoenzymes in human serum and in blood. *Clinica Chimica Acta* **127**, 353-363.

Kato, K.; Ishiguro, Y. & Ariysohi, y. (1983b). Enolase isoenzymes as diseases markers: distribution of three enolase subunits (α, β and γ) in various human tissues. *Dis Markers* **1**, 213.

Keech, C. L. & Brandon, M. R. (1991a). Workshop findings on the ovine homologue of CD5. *Vet Immunol Immunopathol* **27**, 103-107.

Keech, C. L. & Brandon, M. R. (1991b). Workshop findings on the ovine homologue of CD8. *Vet Immunol Immunopathol* **27**, 109-113.

Kelsall, B. L. & Strober, W. (1996). Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J Exp Med* **183**, 237-247.

Kermanizadeh, P.; Hagan, P. & Crompton, D. W. T. (1995). A differential staining method for mast cells and eosinophils in murine intestine, liver and spleen. *Parasitology Today* **11**, 194-196.

Kimambo, A. E. & MacRae, J. C. (1988). Measurement *in vitro* of a larval migration inhibitory factor in gastrointestinal mucus of sheep made resistant to the roundworm *Trichostrongylus colubriformis*. *Vet Parasitol* **28**, 213-222.

Kimambo, A. E.; MacRae, J. C.; Walker, A.; Watt, C. F. & Coop, R. L. (1988). Effect of prolonged subclinical infection with *Trichostrongylus colubriformis* on the performance and nitrogen metabolism of growing lambs. *Vet Parasitol* **28**, 191-203.

Kon, O. M. & Kay, A. B. (1999). T cells and chronic asthma. *Int Arch Allergy Immunol* **118**, 133-135.

Korner, H.; Cook, M.; Riminton, D. S.; Lemckert, F. A.; Hoek, R. M.; Ledermann, B.; Kontgen, F.; Fazekas de St Groth, B. & Sedgwick, J. D. (1997). Distinct roles for lymphotoxin-α and tumor necrosis factor in

organogenesis and spatial organization of lymphoid tissue. *Eur J Immunol* **27**, 2600-2609.

Kriegler, M.; Perez, C.; DeFay, K.; Albert, I. & Lu, S. D. (1988). A novel form of TNF/Cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* **53**, 45-53.

Kroese, F. G.; de Waard, R. & Bos, N. A. (1996). B-1 cells and their reactivity with the murine intestinal microflora. *Semin Immunol* **8**, 11-18.

Lamm, M. E. (1998). Current concepts in mucosal immunity IV. How epithelial transport of IgA antibodies relates to host defense. *Am J Physiol* **274**, G614-G617.

Langkamp-Henken, B.; Glezer, J. A. & Kudusk, K. A. (1992). Immunologic structure and function of the gastrointestinal tract. *NCP* **7**, 100-108.

Lawrence, C. E.; Paterson, J. C.; Higgins, L. M.; MacDonald, T. T.; Kennedy, M. W. & Garside, P. (1998). IL-4-regulated enteropathy in an intestinal nematode infection. *Eur J Immunol* **28**, 2672-2684.

Letterio, J. J. & Roberts, A. B. (1998). Regulation of immune responses by TGF-β. *Ann Rev Immunol* **16**, 137-161.

Liesenfeld, O.; Kang, H.; Park, D.; Nguyen, T. A.; Parkhe, C. V.; Watanabe, H.; Abo, T.; Sher, A.; Remington, J. S. & Suzuki, Y. (1999). TNF-α, nitric oxide and IFN-γ are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with *Toxoplasma gondii*. *Parasite Immunol* **21**, 365-376.

MacDonald, T. T. (1995). Breakdown of tolerance to the intestinal bacterial flora in inflammatory bowel disease. *Clin Exp Immunol* **102**, 445-447.

Mackay, C. (1988). Sheep leukocyte molecules: a review of their distribution, structure and possible function. *Vet Immunol Immunopathol* **19**, 1-20.

Mackay, C. R. (1993). Homing of naive, memory and effector lymphocytes. *Curr Opin Immunol* **5**, 423-427.

MacKay, C. R.; Maddox, J. F. & Brandon, M. R. (1987). A monoclonal antibody to the p220 component of sheep LCA identifies B cells and a unique lymphocyte subset. *Cellular Immunol* **110**, 46-55.

Mackay, C. R.; Maddox, J. F.; Gogolin-Ewens, K. J. & Brandon, M. R. (1985). Characterization of two sheep lymphocyte differentiation antigens, SBU-T1 and SBUT-6. *Immunology* **55**, 729-737.

MacPherson, G.; Jenkins, C. D.; Stein, M. J. & Edwards, C. (1995). Endotoxin-mediated dendritic cell release from the intestine. Characterisation of released dendritic cells and TNF dependence. *J Immunol* **154**, 1317-1322.

Maddox, J. F.; Mackay, C. R. & Brandon, M. R. (1985a). The sheep analogue of leukocyte common antigen (LCA). *Immunology* **55**, 347-353.

Maddox, J. F.; Mackay, C. R. & Brandon, M. R. (1985b). Surface antigens, SBU-T4 and SBU-T8, of sheep T lymphocyte subsets defined by monoclonal antibodies. *Immunology* **55**, 739-748.

Maizels, R. M.; Holland, M. J.; Falcone, F. H.; Zang, X.-X. & Yazdanbakhsh, M. (1999). Vaccination against helminth parasites- the ultimate challenge for vaccinologists? *Immunol Rev* **171**, 125-147.

Manjili, M. H.; France, M. P.; Sangster, N. C. & Rothwell, T. L. (1998). Quantitative and qualitative changes in intestinal goblet cells during primary infection of *Trichostrongylus colubriformis* high and low responder guinea pigs. *Int J Parasitol* **28**, 761-765.

Marangos, P. J.; Schmechel, D.; Pharma, A. M.; Clark, R. L. & Goodwin, F. K. (1979). Measurement of neuron-specific enolase and non-neuronal isoenzymes of enolase in rat, monkey and human nervous tissue. *J Neurochem* **33**, 319-329.

Marangos, P. J.; Zis, A. P.; Clark, R. L. & Goodwin, F. K. (1978). Neuronal, non-neuronal and hybrid forms of enolase in brain: Structural, immunological and functional comparisons. *Brain Res* **150**, 117-133.

March, C. J.; Mosely, B.; Larsen, A.; Cerretti, D. P.; Braedt, G.; Price, V.; Gillis, S.; Henney, C. S.; Kronhein, S. R.; Grabstein, K.; Conlon, P. J.; Hopp, T. P. & Cosman, D. (1985). Cloning, sequencing and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315**, 641-647.

Marrack, P.; Endres, R.; Shimonkevitz, R.; Zlotnik, A.; Dialynas, D.; Fitch, F. & Kappler, J. (1983). The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. *J Exp Med* **158**, 1077-1091.

Martin, L. B.; Kita, H.; Lieferman, K. M. & Gleich, G. J. (1996). Eosinophils in allergy: Role in disease, degranulation and cytokines. *Int Arch Allergy Immunol* **109**, 207-215.

Martins, C.; Mebus, C.; Scholl, T.; Lawman, M. & Lunney, J. (1988). Virus-specific CTL in SLA-inbred swine recovered from experimental African Swine Fever Virus (ASFV) infection. *Ann NY Acad Sci* **532**, 462-464.

Mayer, L. (1997). Review article: Local and systemic regulation of mucosal immunity. *Aliment Pharmacol Ther* **11 Suppl 3**, 81-88.

McClure, S. J.; Davey, R. J.; Lloyd, J. B. & Emery, D. L. (1995). Depletion of IFN-γ, $CD8^+$ or Tcr $\gamma\delta^+$ cells *in vivo* during primary infection with an enteric parasite (*Trichostrongylus colubriformis*) enhances protective immunity. *Immunol Cell Biol* **73**, 552-555.

McClure, S. J.; Davey, R. L.; Emery, D. L.; Colditz, I. G. & Lloyd, J. B. (1996). *In vivo* depletion of T-cells and cytokines during primary exposure of sheep to parasites. *Vet Immunol Immunopathol* **54**, 83-90.

McClure, S. J.; Emery, D. L.; Bendixsen, T. & Davey, R. J. (1998). Attempts to generate immunity against *Trichostrongylus colubriformis* and *Haemonchus contortus* in young lambs by vaccination with viable parasites. *Int J Parasitol* **28**, 739-746.

McClure, S. J.; Emery, D. L.; Wagland, B. M. & Jones, W. O. (1992). A serial study of rejection of *Trichostrongylus colubriformis* by immune sheep. *Int J Parasitol* **22**, 227-234.

McDonald, S. A. C.; Palmen, M. J. H. J.; Van Rees, E. P. & MacDonald, T. T. (1997). Characterisation of the mucosal cell-mediated immune response in IL-2 knockout mice before and after the onset of colitis. *Immunology* **91**, 73-80.

McGowan, I.; Smith, G.-R. & Jewell, D. (1997). HIV infection and the gastrointestinal tract: Advances in mucosal immunology. *Gastroenterol Clin North Am* **26**, 145-173.

Mengheri, E.; Ciapponi, L.; Vignolini, F. & Nobili, F. (1996). Cytokine gene expression in intestine of rat during the postnatal developmental period: increased IL-1 expression at weaning. *Life Sci* **59**, 1227-1236.

Miller, A.; Lider, O.; Roberts, A. B.; Sporn, M. B. & Weiner, H. L. (1992). Suppressor T cells generated by oral tolerisation to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factors β after antigenic-specific triggering. *Proc Natl Acad Sci* **89**, 421-425.

Miller, H. R. (1996). Prospects for the immunological control of ruminant gastrointestinal nematodes: natural immunity, can it be harnessed? *Int J Parasitol* **26**, 801-811.

Miller, H. R. P. (1984). The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Vet Immunol Immunopathol* **6**, 167-259.

Miller, H. R. P.; Jackson, F.; Newlands, G. & Appleyard, W. T. (1983). Immune exclusion, a mechanism of protection against the ovine nematode *Haemonchus contortus*. *Res Vet Sci* **35**, 357-363.

Modlin, R. L.; Mehra, V.; Wong, L.; Fujimiya, Y.; Chang, W.-C.; Horwitz, D. A.; Bloom, B. R.; Rea, T. H. & Pattengale, P. K. (1986). Suppressor T lymphocytes from lepromatous leprosy skin lesions. *J Immunol* **137**, 2831-2834.

Molmenti, E. P.; Ziambaras, T. & Perlmutter, D. H. (1993). Evidence for an acute phase response in human intestinal epithelial cells. *J Biol Chem* **268**, 14116-14124.

Moore, B. W. & McGregor, D. (1965). Chromatographic and electrophorectic fractionation of soluble proteins of brain and liver. *J biol Chem* **240**, 1647-1653.

Mosmann, T. R. & Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* **17**, 138-146.

Mowat, A. M. & Viney, J. L. (1997). The anatomical basis of intestinal immunity. *Immunol Rev* **156**, 145-166.

Murray, A. M.; Simm, B. & Beagley, K. W. (1998). Cytokine gene expression in murine fetal intestine: potential for extrathymic T cell development. *Cytokine* **10**, 337-345.

Nanno, M.; Kanamori, Y.; Saito, H.; Kawaguchi-Miyashita, M.; Shimada, S. & Ishikawa, H. (1998). Intestinal intraepithelial T lymphocytes. Our T cell horizons are expanding. *Immunol Res* **18**, 41-53.

Noguchi, M.; Hiwatashi, N.; Liu, Z. & Toyota, T. (1998). Secretion imbalance between tumor necrosis factor and its inhibitor in inflammatory bowel disease. *Gut* **43**, 203-209.

Ogata, H. & Podolsky, D. K. (1997). Trefoil peptide expression and secretion is regulated by neuropeptides and acetylcholine. *Am J Physiol* **273**, G348-54.

Omer, F. M.; Kurtzhals, J. A. & Riley, E. M. (2000). Maintaining the immunological balance in parasitic infections: a role for TGF-β? *Parasitol Today* **16**, 18-23.

Pahlman, S.; Esscher, T. & Nilsson, K. (1986). Expression of γ-subunit of enolase, neuron-specific enolase, in human non-neuroendocrine tumors and derived cell lines. *Lab Invest* **54**, 554-560.

Pasparakis, M.; Alexopoulou, L.; Episkopou, V. & Kollias, G. (1996). Immune and inflammatory responses in TNF-α-deficient mice: a critical requirement for TNF-α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J Exp Med* **184**, 1397-1411.

Pasparakis, M.; Alexopoulou, L.; Grell, M.; Pfizenmaier, K.; Bluethmann, H. & Kollias, G. (1997). Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor [published erratum appears in Proc Natl Acad Sci U S A 1997 Aug 19;94(17):9510]. *Proc Natl Acad Sci U S A* **94**, 6319-6323.

Pavli, P.; Maxwell, L.; van de Pol, E. & Doe, W. F. (1995). Distribution of human colonic dendritic cells and macrophages-- functional implications. *Adv Exp Med Biol* **378**, 121-123.

Pernthaner, A.; Stankiewicz, M.; Bisset, S. A.; Jonas, W. E.; Cabaj, W. & Pulford, H. D. (1995). The immune responsiveness of Romney sheep selected for resistance or susceptibility to gastrointestinal nematodes: lymphocyte blastogenic activity, eosinophilia and total white blood cell counts. *Int J Parasitol* **25**, 523-529.

Pernthaner, A.; Stankiewicz, M.; Cabaj, W.; Pfeffer, A.; Green, R. S. & Douch, P. G. C. (1996). Immune responsiveness of nematode-resistant or susceptible Romney line-bred sheep to continuous infection with *Trichostrongylus axei*. *Vet Immunol Immunopathol* **51**, 137-146.

Pezzano, M.; Philp, D.; Stephenson, S.; Li, Y.; Reid, V.; Maitta, R. & Guyden, J. C. (1996). Positive selection by thymic nurse cells requires IL-1 β and is associated with an increased Bcl-2 expression. *Cell Immunol* **169**, 174-184.

Pfeffer, A.; Douch, P. G.; Shaw, R. J.; Gatehouse, T. K.; Rabel, B.; Green, R. S.; Shirer, C. L.; Jonas, W. E. & Bisset, S. (1996). Sequential cellular and humoral responses in the abomasal mucosa and blood of Romney sheep dosed with *Trichostrongylus axei*. *Int J Parasitol* **26**, 765-773.

Pinto, A.; Grant, L. H.; Hayes, F. A.; Schell, M. J. & Parham, D. M. (1989). Immunohistochemical expression of neuron-specific enolase and Leu 7 in Ewing's sarcoma of bone. *Cancer* **64**, 1266-1273.

Planchon, S.; Fiocchi, C.; Takafuji, V. & Roche, J. K. (1999). Transforming growth factor-β1 preserves epithelial barrier function: identification of receptors, biochemical intermediates, and cytokine antagonists. *J Cell Physiol* **181**, 55-66.

Planchon, S. M.; Martins, C. A.; Guerrant, R. L. & Roche, J. K. (1994). Regulation of intestinal epithelial barrier function by TGF-β1. Evidence for its role in abrogating the effect of a T cell cytokine. *J Immunol* **153**, 5730-5739.

Poppi, D. P.; MacRae, J. C.; Brewer, A. & Coop, R. L. (1986). Nitrogen transactions in the digestive tract of lambs exposed to the intestinal parasite, *Trichostrongylus colubriformis*. *Br J Nutr* **55**, 593-602.

Pound, J. D. & Gordon, J. (1997). Maintenance of human germinal center B cells in *vitro*. *Blood* **89**, 919-928.

Premier, R. R.; Jacobs, H. J.; Brandon, M. R. & Meeusen, E. N. (1996). Distribution of antigen specific memory T cells in lymph nodes after immunization at peripheral or mucosal sites. *Immunol Cell Biol* **74**, 265-273.

Premier, R. R. & Meeusen, E. N. T. (1998). Lymphocyte surface marker and cytokine expression in peripheral and mucosal lymph nodes. *Immunology* **94**, 363-367.

Press, C.; McClure, S. & Landsverk, T. (1991). Computer-assisted morphometric analysis of absorptive and follicle- associated epithelia of Peyer's patches in sheep foetuses and lambs indicates the presence of distinct T- and B-cell components. *Immunology* **72**, 386-392.

Press, C. M.; Halleraker, M. & Landsverk, T. (1992). Ontogeny of leukocyte populations in the ileal Peyer's patch of sheep. *Dev Comp Immunol* **16**, 229-241.

Puri, N. K.; MacKay, C. R. & Brandon, M. R. (1985). Sheep lymphocyte antigen (OLA). II. Major histocompatibility complex class II molecules. *Immunology* **56**, 725-733.

Ramaswamy, K.; Negrao-Correa, D. & Bell, R. (1996). Local intestinal immune responses to infections with *Trichinella spiralis*. Real-time, continuous assay of cytokines in the intestinal (afferent) and efferent thoracic duct lymph of rats. *J Immunol* **156**, 4328-4337.

Reen, D. J. (1998). Activation and functional capacity of human neonatal CD4 T-cells. *Vaccine* **16**, 1401-1408.

Reinherz, E. L. & Schlossman, S. F. (1980). The differentiation and function of human T lymphocytes. *Cell* **19**, 821-827.

Reynolds, J. D. (1986). Evidence of extensive lymphocyte death in sheep Peyer's patches. I. A comparison of lymphocyte production and export. *J Immunol* **136**, 2005-2010.

Reynolds, J. D.; Kennedy, L.; Peppard, J. & Pabst, R. (1991). Ileal Peyer's patch emigrants are predominantly B cells and travel to all lymphoid tissues in sheep. *Eur J Immunol* **21**, 283-289.

Reynolds, J. D. & Morris, B. (1983). The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur J Immunol* **13**, 627-635.

Reynolds, J. D. & Morris, B. (1984). The effect of antigen on the development of Peyer's patches in sheep. *Eur J Immunol* **14**, 1-6.

Rhind, S. M.; Dutia, B. M.; Howard, C. J. & Hopkins, J. (1996). Discrimination of two subsets of CD1 molecules in the sheep. *Vet Immunol Immunopathol* **52**, 265-270.

Richardson, M.; Beck, C. B. & Clark, D. T. (1968). Prenatal immunisation of the lamb to *Brucella*: dissociation of immunocompetence and reactivity. *J Immunol* **101**, 1363-1366.

Richardson, M. & Conner, G. H. (1972). Prenatal immunisation by the oral route: Stimulation of *Brucella* antibody in foetal lambs. *Infect Immun* **5**, 454-460.

Richardson, M.; Conner, G. H.; Beck, C. C. & Clark, D. T. (1971). Prenatal immunisation of the lamb to *Brucella*; Secondary antibody response *in utero* and at birth. *Immunology* **21**, 795-803.

Rogers, P. A.; Brenton, D. P. & Hopkinson, D. A. (1980). Changes in the activity and isoenzyme patterns of glycolytic enzymes during stimulation of normal lymphocytes with phytohaemagglutinin. *Ann Hum Genet* **43**, 213-226.

Rothel, J. S.; Hurst, L.; Seow, H. F.; Pepin, M.; Berthon, P.; Corner, L. A. & Wood, P. R. (1997). Analysis of ovine IL-1 β production *in vivo* and *in vitro* by enzyme immunoassay and immunohistochemistry. *Vet Immunol Immunopathol* **57**, 267-278.

Rothwell, T. L.; Windon, R. G.; Horsburgh, B. A. & Anderson, B. H. (1993). Relationship between eosinophilia and responsiveness to infection with *Trichostrongylus colubriformis* in sheep. *Int J Parasitol* **23**, 203-211.

Rousset, F.; Garcia, E. & Banchereau, J. (1991). Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J Exp Med* **173**, 705-710.

Sadlack, B.; Lohler, J.; Schorle, H.; Klebb, G.; Haber, H.; Sickel, E.; Noelle, R. J. & Horak, I. (1995). Generalised autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of $CD4^+$ T cells. *Eur J Immunol* **25**, 3053-3059.

Sadlack, B.; Merz, H.; Schorle, H.; Schimpl, A.; Feller, A. F. & Horack, I. (1993). Ulcerative colitis-like disease in mice with a disruptive interleukin-2 gene. *Cell* **75**, 253-261.

Santos, L. M. B.; Al-Sabbagh, A.; Londono, A. & Weiner, H. L. (1993). Oral tolerance to myelin basic protein induces TGF-β secreting T cells in Peyer's patches. *J Immunol* **150**, 115A.

Scheuermann, D. W.; Stach, W.; Timmermans, J.-P.; Adriaensen, D. & Groodt-Lasseel, M. H. A. D. (1989). Neuron-specific enolase and S-100 protein histochemistry for defining the structure and topographical relationship of the different enteric nerve plexus in the small intestine of the pig. *Cell Tissue Res* **256**, 65-75.

Schito, M. L.; Chobotar, B. & Barta, J. R. (1998). Cellular dynamics and cytokine responses in BALB/c mice infected with *Eimeria papillata* during primary and secondary infections. *J Parasitol* **84**, 328-337.

Schmechel, D.; Marangos, P. J.; Zis, A. P.; Brightman, M. & Goodwin, F. K. (1978). Brain enolase as specific markers of neuronal and glial cells. *Science* **199**, 313-314.

Schmechel, D. E.; Brightman, M. W. & Marangos, P. J. (1980). Neurons switch from non-neural enolase to neuron-specific enolase during differentiation. *Brain Res* **190**, 195-214.

Schultz, M.; Tonkonogy, S. L.; Sellon, R. K.; Veltkamp, C.; Godfrey, V. L.; Kwon, J.; Grenther, W. B.; Balish, E.; Horak, I. & Sartor, R. B. (1999). IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation. *Am J Physiol* **276**, G1461-G1472.

Shafer-Weaver, K. A. & Sordillo, L. M. (1997). Bovine $CD8^+$ suppressor lymphocytes alter immune responsiveness during the postpartum period. *Vet Immunol Immunopathol* **56**, 53-64.

Shaw, R. J.; Gatehouse, T. K. & McNeill, M. M. (1998). Serum IgE responses during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *Int J Parasitol* **28**, 293-302.

Shaw, R. J.; McNeill, M. M.; Gatehouse, T. K. & Douch, P. G. (1997). Quantification of total sheep IgE concentration using anti-ovine IgE monoclonal antibodies in an enzyme immunoassay. *Vet Immunol Immunopathol* **57**, 253-265.

Sher, A.; Gazzinelli, R. T.; Oswald, I. P.; Clerici, M.; Kullberg, M.; Pearce, E. J.; Berzofsky, J. A.; Mosmann, T. R.; James, S. L.; Morse, H. C. & Shearer, G. M. (1992). Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol Rev* **127**, 183-207.

Shi, B.-B.; Ishikawa, N.; Itoh, H.; H., I.; Tsuchiya, K.; Horii, Y.; Uchiyama, F. & Nawa, Y. (1994). Goblet cell mucins of four genera of the subfamily *Cricetinae* with reference to the protective activity against *Strongyloides venezuelensis*. *Parasite Immunol* **16**, 553-559.

Shinohara, H.; Semba, R.; Kato, K.; Kashiwamata, S. & Tanaka, O. (1986). Immunohistochemical localisation of γ-enolase in early human embryos. *Brain Res* **382**, 33-38.

Silverstein, A. M.; Uhr, J. W.; Kraner, K. L. & Lukes, R. J. (1963). Foetal responses to antigenic stimulus: II Antibody production by the foetal lamb. *J Exp Med* **117**, 799-813.

Smith, K. A. (1992). Interleukin-2. *Curr Opin Immunol* **4**, 271-276.

Specian, R. D. & Oliver, M. G. (1991). Functional biology of intestinal goblet cells. *Am J Physiol* **260**, C183-C193.

Steel, J. W. & Symons, L. E. A. (1982). Nitrogen metabolism in nematodiasis of sheep in relation to productivity. In *Biology and control of endoparasites*, pp. 235-256. Edited by L. E. A. Symons; A. D. Donald & J. K. Dineen. Sydney: Academic Press.

Steel, J. W.; Symons, L. E. A. & Jones, W. O. (1980). Effects of level of larval intake on the productivity and physiological and metabolic responses of lambs infected with *Trichostrongylus colubriformis*. *Aust J Agric Res* **31**, 821-838.

Stevenson, L. M.; Huntley, J. F.; Smith, W. D. & Jones, D. G. (1994). Local eosinophil and mast cell-related responses in abomasal nematode infections of lambs. *FEMS Immunol Med Microbiol* **8**, 167-173.

Stewart, M. L.; McClure, S. J.; Rothwell, T. L. W. & Emery, D. L. (1995). Changes in the enteric innervation of the sheep during primary or secondary immune responses to the nematode *Trichostrongylus colubriformis*. *Reg Immunol* **6**, 460-470.

Strobel, S. & Mowat, A. M. (1998). Immune responses to dietary antigens: oral tolerance. *Immunol Today* **19**, 173-181.

Sun, J.; Dirden-Kramer, B.; Ito, K.; Ernst, P. B. & Van Houten, N. (1999). Antigen-specific T cell activation and proliferation during oral tolerance induction. *J Immunol* **162**, 5868-5875.

Svetic, A.; Madden, K. B.; Zhou, X. D.; Lu, P.; Katona, I. M.; Finkelman, F. D.; Urban, J. F., Jr. & Gause, W. C. (1993). A primary intestinal helminthic infection rapidly induces a gut- associated elevation of Th2-associated cytokines and IL-3. *J Immunol* **150**, 3434-3441.

Swain, S. L. (1983). T cell subsets and recognition of MHC class. *Immunol Rev* **74**, 129-142.

Swain, S. L.; Bradley, L. M.; Croft, M.; Tonkonogy, S.; Atkins, G.; Weinberg, A. D.; Duncan, D. D.; Hedrick, S. M.; Dutton, R. W. & Huston, G. (1991). Helper T-cell subsets: Phenotype, function and the role of lymphokines in regulating their development. *Immunol Rev* **123**, 115-144.

Tartaglia, L. A.; Weber, R. F.; Figara, I. S.; Reynolds, C.; Palladino, M. A. J. & Goeddel, D. V. (1991). The different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* **88**, 9292-9296.

Tierney, T. J.; Trevella, W. & Simpson-Morgan, M. W. (1997). The cellular response of lambs to *Brucella ovis* before and after birth. *Vet Immunol Immunopathol* **59**, 369-373.

Toellner, K.-M.; Scheel-Toellner, D.; Sprenger, R.; Duchrow, M.; Trumper, L. H.; Ernst, M.; Flad, H.-D. & Gerdes, J. (1995). The human germinal centre cells, follicular dendritic cells and germinal centre cells produce B cells-stimulating cytokines. *Cytokine* **7**, 344-354.

Tremain, S. A. (1993). TITRECAL: an MS-DOS program for automated calculation of antibody titres from ELISA data. *J Immunol Methods* **166**, 295-296.

Urban, J. F. J.; Katona, I. M. & Finkelman, F. D. (1991). $CD4^+$ but not $CD8^+$ T cells regulate the IgE response and protective immunity in mice. *Exp Parasitol* **73**, 500-511.

Vassalli, P. (1992). The pathophysiology of tumor necrosis factors. *Ann Rev Immunol* **10**, 411-452.

Verdugo, P. (1990). Goblet cells secretion and mucogenesis. *Annu Rev Physiol* **52**, 157-176.

Viney, J. L.; Mowat, A. M.; O'Malley, J. M.; Williamson, E. & Fanger, N. A. (1998). Expanding dendritic cells *in vivo* enhances the induction of oral tolerance. *J Immunol* **160**, 5815-5825.

Wagland, B. M.; Emery, D. L. & McClure, S. J. (1996). Studies on the host-parasite relationship between *Trichostrongylus colubriformis* and susceptible and resistant sheep. *Int J Parasitol* **26**, 1279-1286.

Waters, W. R.; Harp, J. A. & Nonnecke, B. J. (1995). Phenotypic analysis of peripheral blood lymphocytes and intestinal intraepithelial lymphocytes in calves. *Vet Immunol Immunopathol* **48**, 249-259.

Weiner, H. L. (1997). Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today* **18**, 335-343.

Weiner, H. L.; Friedman, A.; Miller, A.; Khoury, S. J.; Al-Sabbagh, A.; Santos, L.; Sayegh, M.; Nussenblatt, R. B.; Trentham, D. E. & Hafler, D. A. (1994). Oral tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune disease by oral administration of autoantigens. *Annu Rev Immunol* **12**, 809-837.

Wershil, B. K. & Walker, W. A. (1992). The mucosal barrier, IgE-mediated gastrointestinal events, and eosinophilic gastroenteritis. *Gastroenterol Clin North Am* **21**, 387-404.

Whary, M. T.; Zarkower, A.; Confer, F. L. & Ferguson, F. G. (1995). Age-related differences in subset composition and activation responses of intestinal intraepithelial and mesenteric lymph node lymphocytes from neonatal swine. *Cell Immunol* **163**, 215-221.

Whitacre, C. C.; Gienapp, I. E.; Orosz, C. G. & Bitar, D. M. (1991). Oral tolerance in experimental autoimmune encephalitis. *J Immunol* **147**, 2155-2163.

Whitlock, H. V. (1948). Some modifications of the McMaster helminth egg-counting technique and apparatus. *J Council for Scientific and Industrial Res* **21**, 177-179.

Winter, M.; Wright, C.; Blakeley, D. & Lee, D. (1995). Mast cells and eosinophil staining. *Parasitology Today* **11**, 426.

Wood, P. R. & Seow, H.-F. (1996). T cell cytokines and disease prevention. *Vet Immunol Immunopathol* **54**, 33-44.

Woywodt, A.; Ludwig, D.; Neustock, P.; Kruse, A.; Schwarting, K.; Jantschek, G.; Kirchner, H. & Stange, E. F. (1999). Mucosal cytokine expression, cellular markers and adhesion molecules in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* **11**, 267-76.

Wyatt, C. R.; Brackett, E. J.; Perryman, L. E.; Rice-Ficht, A. C.; Brown, W. C. & O'Rourke, K. I. (1997). Activation of intestinal intraepithelial T lymphocytes in calves infected with *Cryptosporidium parvum*. *Infect Immun* **65**, 185-90.

Yang-Snyder, J. A. & Rothenberg, E. V. (1998). Spontaneous expression of interleukin-2 *in vivo* in specific tissues of young mice. *Dev Immunol* **5**, 223-245.

Yap, K. L.; Ada, G. L. & McKenzie, I. F. C. (1978). Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* **273**, 238-239.

Youinou, P.; Jamin, C. & Lydyard, P. M. (1999). CD5 expression in human B-cell populations. *Immunol Today* **20**, 312-316.

Zeitz, M.; Greene, W. C.; Peffer, N. J. & James, S. P. (1988). Lymphocytes isolated from the intestinal lamina propria of normal nonhuman primates have increased expression of genes associated with T- cell activation. *Gastroenterology* **94**, 647-55.

Zhang, K. Z.; Westberg, J. A.; Holtta, E. & Andersson, L. C. (1996). BCL2 regulates neural differentiation. *Proc Natl Acad Sci U S A* **93**, 4504-4508.